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WORKBOOK ON CONVALESCENT BURN SERUM

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DEPARTMENT OF SURGERY

THE UNIVERSITY OF TEXAS MEDICAL BRANCH

GALVESTON, TEXAS

WORKBOOK ON CONVALESCENT BURN SERUM

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Galveston, Texas

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This report is one of five publications resulting from a study on "Evaluation of Burn Serums and Treatment of Mass Burn Casualties," prepared under the direction of The Division of Health Mobilization, Public Health Service, Department of Health, Education, and Welfare:

1. Simplified Standardized Treatment of Burns...
2. Workbook on Convalescent Burn Serum
3. Newer Trends in Burn Research...
4. New Concepts in Burn Physiology and Burn Treatment
5. Burns Bibliography 1952 - 1962

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INTRODUCTION

INTRODUCTION

The present status of the Convalescent Burn Serum and Burn Toxin issues has been reviewed in a recent issue of the _Annals of Surgery_ by the group at the University of Texas Medical Branch in a paper entitled "Experimental Studies with Reference to Antigen-Antibody Phenomena following Severe Extensive Burns." A copy of this publication is enclosed together with other material which is not readily available to the reviewer, for one reason or another, as a supplement to articles in the recent literature and to the rather extensive bibliographies which have been compiled in the past.

The transactions of the 1960 First International Congress on Research in Burns, which were published in 1962 as _Research in Burns_, edited by Curtis P. Artz (A. I. B. S. Pub. No. 9, Washington), included six papers on Toxins and Convalescent Serum:

Chaet, Alfred B.   Demonstration of Burn Toxins in Invertebrates

Simonart, A. J. L.   Survival after Lethal Burn of Previously Treated Rabbits

Dobrkovsky, M., Dolezalova, J., and Pavkova, L. Immunological and Biochemical changes in Burns

Feodorov, N. A. and Skurkovich, S. V. Immunotherapy of Burn Sickness

Rosenthal, Sol Roy, Hartney, James B., and Spurrier,
Wilma A. Tissue Culture and
Serological Demonstrations of "Toxin-
Antitoxin" Phenomenon in Injury

Malm, Ole J. and Slawikowski, George J. M. Evaluation
of Different Types of Convalescent
Burn Sera in the Rat

Except for Malm's study, which represented a preliminary report, the

majority of these presentations covered previously published data.

The N. R. C. workshop on <u>Immunotransfusion in the Treatment of Burns</u>

held in August, 1961, with Dr. Gannon and Dr. Pennell of the Sub-

committee on Plasma serving as co-chairmen, reviewed current

laboratory and clinical investigations and published a report in November,

1961, with a summary of findings and a number of appendices of pertinent

auxiliary material, including for the most part unpublished reports of

work in progress. Because of wide interest in the subject and limited

circulation, it is now out of print, and for this reason permission has

been obtained from the proper authorities to duplicate this pamphlet

together with unpublished monographs of Sell at the Tissue Bank, Naval

Medical Center, Bethesda, and of Malm and Slawikowski at Walter

Reed Army Medical Center.

Since the presentation of Schütz of Austria at the Fifteenth International

Physiology Congress in Leningrad in 1935 may have been the inspiration for later investigations with convalescent burn sera and burned skin extracts, a translation of the abstract of his paper has been prepared. Results in twice-burned animals are of particular interest.

Finally, in attempting to explain the precipitation lines obtained in Ouchterlony plate studies by Chambler in our laboratory working with boosted convalescent sera, the article in Nature with regard to "Spurious 'Auto-Immune' Reactions" was encountered. This is quoted in its entirety for reference purposes.

EXPERIMENTAL STUDIES WITH REFERENCE TO

ANTIGEN-ANTIBODY PHENOMENA

FOLLOWING SEVERE EXTENSIVE BURNS

Peter Matter, M. D.,   Kenneth Chambler, M. D.,

Bruce Bailey, M. D.,   S. R. Lewis, M. D.,

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Clinical trial of convalescent burn serum, which followed the experi-
mental studies of Feodorov and his co-workers [10], has stimulated

considerable interest in recent years in reappraisal of the problem of

whether or not toxemia following thermal trauma is a specific entity and,

if so, whether it occurs as an immune phenomenon or is related to

other causative factors. Meanwhile in spite of Soviet [11] and Czech [8]

experience and occasional reports of therapeutic use of the serum in

other areas, including a few cases following the Chicago School fire [28],

it was concluded in November, 1961 at a workshop conference on Immuno-

transfusion in the treatment of burns sponsored by the Subcommittee on

Plasma, the National Academy of Sciences, National Research Council [15],

that available data do not indicate whether or not convalescent serum,

blood, or plasma surpass other methods of treatment of acute burns to a

statistically reliable degree.

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Should the concept of immunotransfusion be valid, it was further
questioned whether the benefits of such therapy might not in fact be
related to antibodies against antigens of bacterial origin rather than
to specific substances produced in heat-damaged tissues, including
elements of the blood. Finally, the point was raised that the published
mortality figures on burns in the Soviet Union by Pushkar[24], Petrov[23], and
others indicate that death during the first 48 hours following injury is
still a major problem in recognized burn centers as it was in this
country 20 years ago, and that more variables might exist to confuse
the issue than were apparent at first glance.

Sevitt[36] has discussed the background of burn toxemia in detail and Malm,
who has completed an historical review of the subject with 234 referen-
ces in a monograph as yet unpublished[17], summarized his findings at
the 1960 International congress on Research on Burns[18], reporting as
the earliest references Wertheim's work in 1868[40, 41], and Avdakoff's[2] in
1876, both of whom studied the effects of injections of blood from burned
into unburned animals. By 1925 more than 1,000 references had been
collected by Harkins[14] in his Textbook on burns, but with developments
in surgical physiology and bacteriology, articles both "pro" and "con"
began to appear more infrequently in the literature. Brancati[7] in 1923
had advanced the theory of an antigenic toxin elaborated in burned tissue

on the basis of anaphylactic shock in guinea pigs subjected to small experimental burns, and this concept was developed by Simonart[39] and a number of workers including Sol Ray Rosenthal,[26, 27] who in 1937 reported both a specific burn toxin and a neutralizing antitoxin. Schutz[32] in 1935 at a meeting of the Fifteenth International Congress in Leningrad mentioned what appears to be the first therapeutic use of convalescent serum and summarized an interesting series of experiments in twice-burned animals. Subsequently Segal and Uzdin[33] in 1940 published the results of 4 years experience with convalescent serum in clinical patients. In the middle 1950's there appeared the work of Feodorov and Skurkovich,[10] Simonart's article on auto-intoxication after burns,[39] and additional publications by Rosenthal and his co-workers.[29, 30]

The Soviets demonstrated <u>in vivo</u> evidence for specific antigens produced from skin injured by thermal trauma and for the development of burn toxin antibodies as an autoimmunization phenomenon. Guinea pigs sensitized by extracts of burned skin were demonstrated with normal serum and skin extracts and then subjected to further injection of burned skin extract with resultant anaphylactic shock.

The antigen was characterized as being a thrombin-like material, heat-labile, incapable of passing through a Seitz Filter, and not species-specific.

In the presence of antigen from burned skin, serum of burned dogs showed, according to these workers, an ability to fix complement, by the prolonged cold complement fixation technique, whereas tests with normal skin extracts from the same animals were weak or negative. Complement fixation tests became positive about the 7th post-burn day and reached a maximum titer between 20 and 40 days. Activity of the serum could be destroyed on heating to 65° C. for 30 minutes.

Following standardized burns in unanesthetized dogs produced by flaming alcohol sponges it was also reported that improvement in toxemic symptoms and in early mortality was obtained from the use of convalescent burn serum and "iso-immune" serum taken from animals who had received repeated injections of blood from acutely burned dogs. As a result of these laboratory experiments convalescent burn serum was adopted in the USSR for routine therapeutic use in a number of institutes treating acute burns.

Simonart's studies included injection of in vitro heat-denatured serum proteins and of commercial preparations of polypeptides into laboratory animals. No toxic reactions were noted on intravenous injections, but morbid or lethal effects occurred on subcutaneous administration into rabbits or into the ventral lymph sac of the frog. It was his theory that

the toxicity resulted from hydrolysis by a proteolytic enzyme present in lymph fluid, and he noted that the euglobulin factor extracted from peptone-produced edema provoked the same results when injected into other animals. This study and that of Godfraind,[12] working with Simonart, who advanced the hypothesis that the pathological changes in acute burns were related to increased protease activity, have been challenged in particular by Allgower,[1] who failed to duplicate their results under conditions of rigid bacteriological control. Allgower has, however, emphasized the fact that greater "toxicity" results when animals are subjected to higher levels of thermal damage and has reported that blood heated to 96° C. produces a 30% mortality in rabbits within 48 hours when injected intraperitoneally (50% in eight days), whereas no morbidity is noted from blood heated to 80° C. Whether or not specific toxic agents are involved is still a moot question.

Rosenthal and his associates in a series of publications including a presentation at the International Congress on Research in Burns,[31] have reported evidence for the presence of toxins in acute burn sera as demonstrated by inhibition of HeLa cell tissue culture growth, hemolysis of red cells of acutely burned individuals, and precipitinogens against healed burn sera. These effects have been neutralized by "antitoxins" present in the blood of healed burned individuals. Graber[13] at the Surgical

Research Unit at the Brooke Army Medical Center, utilizing Rosenthal's techniques with the HeLa cell cultures, was unable to duplicate his results. Sell,[34] at the Tissue Bank of the U.S. Naval Medical School in Bethesda, and Miller,[19] at the Naval Medical Research Unit at Great Lakes, in a controlled double-blind study of a large number of specimens in 1961, found confirmation only when there was hemolysis of specimens or when there had been exposure to sunlight. Since Rosenthal's technique included storage of the serum with the clot and since controlled data had been impossible to obtain during the study of the Chicago School fire patients, Miller has stated that no positive conclusions can be drawn from the work conducted in his laboratory at that time and that data obtained were "consistent with" but "in no way confirmatory of a toxin-antitoxin concept." Meanwhile, at the Burns Unit in Prague under the direction of Dobrkovsky, Pavkova and Dolesalova, clinical studies have been in progress for several years. Convalescent burn serum is employed in clinical subjects, and clinical signs and symptoms are correlated with a characteristic curve of serum antibody levels employing a collodion agglutination method against antigens obtained from both involved and uninvolved skin of burn patients. These are believed to be disintegration products of a polypeptide or mucopolysaccharide nature.

Malm and his associates [17, 18] have studied extensively the effect of convalescent serum in burned rats, and his experiences have paralleled rather closely those of our laboratory,[6] with preliminary enthusiasm for convalescent burn serum dampened by later failure to obtain significant differences in survival rates in the early period. In his case when a change was made to pathogen-free rats at the Walter Reed Army Institute of Research, a high enough mortality rate could not be obtained in the control series against which to evaluate the immediate effects of convalescent serum therapy although slight protective effects were noted in terms of ultimate survival when very extensive burns were inflicted.

Sanford Rosenthal[25] at the Peru Project in Lima has reported that use of convalescent serum in burned mice has produced results "only slightly more effective" than normal gamma globulin. Koslowski,[16] in investigating the same problem in burned rats, has found no improvement in overall mortality.

Recent attempts by Moyer's group, by Sell, and by our laboratory to reproduce the original experiments of Feodorov in guinea pigs have been unsuccessful. Although delayed anaphylactoid reactions have been noted, no true anaphylaxis has been observed.

Additional facets of investigation with reference to antigen-antibody

phenomena following thermal trauma, which have given inconclusive and
inconsistent results, have included the use of Schults-Dale tests by
Chambler and Matter in our group and by Sell;[35] serological techniques
by Graber[13] and by Chambler, and gel-precipitation (Ouchterlony plates)
and immunoelectrophoretic studies by Chambler and Matter which have
been confirmed by Sell.[35] Matter has also utilized a latex particle fixation
test as a substitute for the collodion particle solution of Pavkova in an
effort to demonstrate antibody titers to burned skin in the serum of clini-
cal patients.

Soon after the publication of Feodorov's work a series of studies was
carried out in our laboratory employing exchange transfusions from
acutely burned dogs into normal animals. Results were rather inconsis-
tent in terms of mortality and toxic symptoms, and the experiments were
abandoned for lack of suitable monitoring devices. The present investi-
gations were initiated in 1960.

Experiences on our service at the University of Texas Medical Branch
during the past two years may be summarized as follows:

In Vivo Studies

In an effort to study the therapeutic effects of convalescent burn serum in

rats, before directing attention to the clinical patient, a series of experiments was set up originally similar to those of Malm and his co-workers. These were conducted by Bailey, who had previously devised and tested instrumentation for standardized burns in which the degree of body weight immersion could be correlated with the extent of burn inflicted by hot water with great accuracy, the two functions of dipping-and-weighing and dipping-and-burning being separated in the process.

In experiments which involved several thousand animals published by Bailey in 1961[3], it was noted that standard scalds of 65% weight immersion at 90° C. for 15 seconds in 200 gm. female Holtzman albino rats resulted in 100% mortality between 20 and 40 hours with an average of 27 hours, discarding anesthetic deaths from Nembutal. In convalescent-serum treated groups the average survival time was 60 hours with a range, for the most part, of 40 to 80 hours. The convalescent serum was prepared from animals which had survived scalds at 55% of weight immersion (approximately 35% surface area) at 90° C. for 15 seconds inflicted 3 months previously - a group of 100 out of 1,000 animals. Serum was prepared after intravenous injection of 20 cc. of normal saline containing 5,000 units of penicillin and 5 mg. of streptomycin and was stored in sterile flasks at -10° C. The significance of this technique has not been evaluated, but sera drawn at shorter intervals following

burning, 2 to 8 weeks, proved ineffective in prolonging early mortality.

Intravenous injections of crude extracts of both normal and burned skin into healthy animals were immediately fatal; subcutaneous injection of scalded skin extract and intravenous administration of 5 cc. of acute burn serum produced variable results, toxic as a rule, rather than lethal.

Additional experiments were conducted by Bailey to assess the effect of prior scalding on subsequent re-burning of the laboratory rats, other types of trauma being employed for control purposes. Results suggested that this type of "pretreatment" prolonged ultimate survival and indicated some protective influence as a simple response.

In the course of further in vivo experiments under Chambler with convalescent burn serum in Holtzman rats it was noted that a group of control animals not only survived longer than 6 days but that many lived longer than those treated with convalescent serum. Since the deaths in the latter series were suspected of being related to the anesthesia, Nembutal 3 mgm./100 gm., and since animals had been discarded previously because of failure to regain consciousness following experimental burns, it was decided to change from barbiturate to ether anesthesia. At the same time animal quarters were moved, and with warmed

temperatures of 22° to 24° C. and in a draft-free room, without an air-conditioning unit in the vicinity, the rats used as controls lived for longer periods of time than previously. It was therefore decided to evaluate first the factors in the laboratory which might have affected the overall burn mortality.

Experiment I

Two groups of 10 female Holtsman rats weighing 200 gm. $\pm$ 10 were given a standard burn of 65% of body weight immersion in water of 90° C. for 20 seconds. Group A was anesthetized with pentobarbitol, Group B with ether. The rats were kept in separate cages with water and food ad lib. in a room with a constant temperature of 23° C. The animals of the second group recovered from their ether anesthesia in less than 15 minutes, some of them drinking water by this time. None of the pentobarbitol anesthetized rats recovered earlier than 90 minutes after the burn. Two rats in this group died during the first six hours without regaining consciousness after the burn.

Rectal temperature measured by thermocouple showed an average decrease 3 hours post burn from 36 to 32° C. in Group A in contrast to an average drop of only one degree C. in Group B. The average fluid intake in the first 24 hours was 28 cc. in Group A and 48 cc. in

Group B which indicated that prolonged recovery time from anesthesia was a definite factor in post burn therapy.

Since all remaining 18 rats survived for a prolonged period of more than 60 hours, it was assumed that results obtained previously were due in part to the effect of the anesthetic pentobarbitol and partly to the influence of the immediate environment with respect to temperature and draft.

In order to observe the influence of convalescent serum on mortality following standardized burns under ether anesthesia and in warmer environment, Bailey's experiments were next repeated using first the remaining convalescent serum from his studies and later serum collected after three months from survivors of rats subjected to a 40% weight immersion burn (about 32% of the body surface area) in hot water of 90° for 20 seconds. The animals were exsanguinated by a carotid cut without prior injection of saline and antibiotics.

Experiment II

Female Holtzman rats of 170 to 180 grams were epilated and anesthetized with ether in a glass chamber until unconscious. As soon as they were attached to the rat frame on the burn machine anesthesia was

reinforced with open-drop ether applied by a cotton wool pad. All experimental rats were awake and moving five to ten minutes following burning. The following categories were studied in groups of 10 animals each:

Group 1. Burning, no therapy except food and water ad lib (controls).

Group 2. Burned rats treated with three cc. of dextrose-saline intraperitoneally immediately after the burn and at four and eight hours. Food and water ad lib. (controls).

Group 3. Burned rats treated as in Group 2 with the addition of 1.5 cc. of normal rat serum given intravenously very slowly immediately post burn (controls).

Group 4. Burned rats treated as in Group 2 with addition of 1.5 cc. of convalescent burn serum administered as in Group 3, slowly and immediately post burn.

Results: In Group 1 controls, five animals (50%) survived longer than six days. Death occurred in these at five, seven, eight, ten, and 38 hours after the burn. In Group 2 controls, there were only three survivors (30%) after six days. Times of death were at three, four, five, 22, 24, 38 and 53 hours. In Group 3 controls, were five six-day survivors (50%) with deaths as follows: 20 hours (due to hemoperitoneum); 29 hours; 45

hours (due to pneumonia); 59 hours; and 5 days.

In Group 4 were eight survivors (80%); one death occurred 70 hours post burn and one at 5 days.

It was obvious from this study that early mortality differed from results obtained in Bailey's experiments; · as a matter of fact, a large number of the animals survived indefinitely, irrespective of initial therapy. Since two of the deaths in Group 3 could be explained sufficiently by post mortem findings aside from specific effects of the burn, it was believed that there was insufficient difference between Groups 4 and 5 to demonstrate any evidence of a protective effect of convalescent serum. Subsequent repetition of these studies showed essentially the same results -- approximately 50% mortality at the end of a week or 10 days, regardless of the type of therapy. Since late deaths·were associated with local infection or with immobility from constricting effect of the burn eschar which resulted in inadequate intake of fluids and food, it was felt that the test was not suitable for estimation of the influence of the sera on ultimate survival and that if a specific burn toxin should exist, it must be related to morbid rather than lethal factors, at least under the conditions of the experiment with Holtzman albino rats in the indicated weight range.

Efforts were directed, next, at producing a more severe burn which
would result in a high mortality during the early post burn period and
furnish a test animal for evaluation of the protective effects of convales-
cent serum and "immune" serum. For this purpose a series of experi-
ments was conducted in which rats with burns of 30%, 45% and 60%
were exposed to scalds at 90° C. for periods of time varying from
15 to 45 seconds. Previous studies made in association with develop-
ment of the Bailey Burner had shown that a 65% weight immersion scald,
corresponding to approximately 50% of body surface in extent, was
the feasible upper limit which could be utilized without involving the
genitalia, the head and neck, or the extremities of the animal. At
15 seconds all animals survived in all categories; at 45 seconds all
animals died, including those treated with normal and convalescent
serum, indicating either that the burn inflicted for this length of ex-
posure was overwhelming or that convalescent serum had no pro-
tective effect. Below 45 seconds no predictable results could be ob-
tained in the controls.

Finally, as a preliminary to in vitro tests limited experiments were
conducted to attempt to confirm the anaphylaxis experiments of Feo-
dorov in both rabbits and guinea pigs, sensitizing with antigen source,
(rat burn serum) desensitizing, and subsequently testing animals with

injection of antigen and to re-evaluate the effect of injections of burned and normal skin extracts which Bailey had reported. In the first series, animals became ill and a number of the guinea pigs had delayed anaphylactoid deaths, but no true anaphylaxis was observed and post mortem examinations were negative. In the second series intraperitoneal, subcutaneous and intravenous injections of skin extracts were employed in the following experiment:

Experiment III

Aqueous extracts were made from normal rat skin and from animals subjected to a 50% burn 18 hours previously. Two burned skin extracts prepared from animals already dead for some hours were discarded, and a third was taken from a moribund animal for testing purposes. Extracts were prepared by mincing, centrifuging and simple filtration with paper filters and water suction only.

    a. Injection of 1 cc. of normal and burned skin extracts with 1 cc. of normal saline had no effect when injected intraperitoneally into a number of healthy rats

    b. Four previously healthy rats were injected subcutaneously with 1 cc. of burned skin extract and 1 cc. of normal saline mixed. Three of the rats died within 60 seconds. The other rat became inert

immediately and then tremulous and convulsive and in one to two minutes was unconscious, but no anaphylactic signs were noted. Over a period of three hours it gradually improved but was found dead 16 hours later. A fifth rat was given 1 cc. of burned skin extract mixed with 1 cc. of "immune" serum from a rat which had recovered from a severe burn six weeks previously. No ill effects were noted following this injection nor after a subsequent injection in the same dosage.

c. The same burned skin extract was diluted to half-strength with saline, and 2 cc. were injected intravenously into both normal and previously burned rats. In both instances the extract had an immediate lethal effect. When injected into 2.5 kgm. rabbit, however, death did not result.

d. The extract was cultured for bacteria; tested for hypo- or hypertonicity; and analyzed with respect to alterations in protein content in comparison with normal rat skin and for variations in sodium and potassium content. All tests were normal, and in addition, intradermal sensitivity tests were conducted in the rat which had recovered following the convulsive episode. Results were negative to both normal and burned skin extract after 24, 48 and 72 hours.

e. It was planned to centrifuge the extract further in order

to minimize particulate emboli and then to separate the material into its albumin and globulin components for further testing, but following further clearing of the filtrate the extract proved to be ineffective in producing toxic symptoms when injected either subcutaneously or intra- venously.

Morbid and lethal effects of injections of extracts of normal and burned skin noted in the first part of the experiment and apparently also in Bailey's work must be concluded, hence, to be the result of technical failure initially to clear the extracts of particulate matter. This experiment does not furnish evidence, however, refuting the existence of non-toxic burn antigens in burned tissue.

In Vitro Studies

Attention was next directed toward in vitro experiments, in which both Chambler and Matter have participated.

Assuming the existence of circulating antibodies in the serum of subjects recovering from severe thermal trauma -- aside from those which have developed in response to antigens associated with bacterial infection -- a series of studies was undertaken in an effort to demon- strate the presence of specific burn antigens which might be impli-

cated as toxic factors. It was recognized at the outset that although in vitro tests might verify the existence of an antigen-antibody reaction, no information could be obtained as to the type of pathological process involved, or indeed to the existence of pathology.

From available evidence it was considered that any specific toxin must arise from a deep burn, probably full-thickness in degree; that the toxin produced from coagulated burned tissue would be of protein origin or at least a substance capable of eliciting an immune response; and that the altered or new protein should be toxic to the host when absorbed. It was recognized also that the studies would be affected by the mechanism of clearance of absorbed toxin from the circulating blood, i.e., by cell fixation, detoxication, or renal excretion, and the time of clearance, whether early or late, as well.

Initially, homologous and heterologous sera tests were conducted as follows:

Experiment IV

Homologous serum was collected by exsanguination under ether anesthesia of female Holtzman rats which had completely recovered and healed from a 30% weight immersion hot-water burn at 90°C. for

15 seconds. Blood was allowed to stand overnight, after which the
serum was gently drawn off from the clot. Small amounts of peni-
cillin and streptomycin were added to this pooled primary-response
or unboosted serum. Secondary-response or boosted serum was pre-
pared by the same technique 12 days after re-burning at 90° C. for
20 seconds (20% weight immersion) a rat which had recovered from
a 30% burn as above. This boosted serum presumably should con-
tain a higher concentration of specific antibodies against burn toxin.

Materials to be tested for the presence of antigens included aqueous
extracts of burned rat skin prepared with unbuffered saline and ex-
cised at one hour and 24 hours post burn; acutely burned rat sera,
with animals sacrificed at the time of burning and at four minutes,
eight minutes, 12 minutes, 30 minutes, one hour, four hours, eight
hours, 12 hours, 24 hours and 48 hours; and diffusion products of rat
muscle. Normal skin extracts and and normal serum were employed
as controls.

Heterologous sera were prepared in rabbits by sensitization with
1) normal rat sera, to be used for control purposes; 2) serum collected
from rats immediately after subjecting them to a standardized burn;
and 3) rat serum collected 48 hours after burning. Rabbits were immu-

nised by injection of .5 cc. of serum at 2-day intervals x 3 and then repeating the procedure after an interval of one week.

a. Hemolysin reaction. An increased hemolytic effect was demonstrated against washed rabbit cells (1/60 suspension between 1/2 and 1/32 dilution, using non-inactivated burn rat serum.) This phenomenon has been noted by other workers, in particular by Sell and Graber, with whom results were checked.   Its significance is not known.   There is, however, a tendency toward hemolysis of normal rabbit cells by normal rat serum which seems to be reinforced in some manner by thermal trauma.

b. Agglutination reactions. Numerous experiments were conducted over a period of several months with Boyden's Tanned Cell. Inconsistent results were obtained, and as the test was eventually abandoned because of technical difficulties, no information was obtained as to the presence of agglutinin antigens in burned sera by this method.

Since the reagents employed by Pavkova for the collodion particle tests were not available in this country, an attempt was made next to duplicate her results, substituting Latex Particles in a test which had been employed by Singer and Plotz[38] for the serologic diagnosis of rheumatoid arthritis for collodion particles. The tissue to be used

as antigen was homogenized with 1.1% NaCl and frozen. As required it was thawed, centrifuged, and the supernatant was filtered with a Seitz filter and used as antigen. The protein content was determined by absorbence at 280 mu and the equivalent of 2.5 mg. was diluted to 10 cc. with borate-saline buffer, pH 8.2 and mixed with 0.1 cc. stock latex solution (Dow polystyrene latex) 0.81 microns in size. The serum to be tested was diluted serially from 1:2 to 1:520 with borate-saline buffer. 0.5 cc. of the antigen suspension was added to 0.5 cc. of diluted serum, and the mixture was incubated at 37° for 18 to 24 hours. The tubes were then centrifuged at 2,300 rpm. for ten minutes at 5° C. The agglutination indicating a positive test was easily observed by tapping gently on the tubes to resuspend the latex particles. Plain latex without antigen was employed for control.

a. Burned and unburned skin was taken from three patients within 12 hours after injury and used as antigen. No titer could be demonstrated in the serum.

b. Using the same technique, pre-and-post-burn lymph was collected from the left thoracic duct of three dogs immediately following administration of a standardized burn and at intervals during the first 24 hours in a clinical burn patient as well.

Beginning on the fifth day after injury and twice weekly thereafter serum samples were tested by the latex fixation method, but no significant titers could be detected which were related to the burn per se. In one dog which had a clinical wound infection, bacterial titers were obtained which indicated that this method is sensitive and reliable.

c. Precipitation reactions. For diffusion-in-gel tests (Ouchterlony plates) a seven-well diffusion pattern as described by Feinberg[9] was prepared with a 1/2% special Noble Agar. A buffer was not included but 200 mgm. of 1% methiolate powder was added as a bacteriostatic agent. These reagents were dissolved by stirring in a boiling water bath for 15 minutes. Polished petri dishes previously sprayed with silicone were then filled with 20 cc. of the agar solution.

Empty Ouchterlony plates were refrigerated whereas charged plates were kept at room temperature in a chamber of high humidity.

Homologous sera were collected by exsanguination of female Holtzman rats which had recovered completely healed from a 40% weight immersion scald burn at 90° C. for 20 seconds. One hundred thousand units of penicillin was added to the serum. Boosted serum was prepared by

the same technique 12 days after reburning rats which had recovered
from a 40% burn, as above, in order to obtain a higher concentration
of any specific antibodies against burn toxin which might be present.

Materials to be tested for the presence of antigen included aqueous
extracts of burned rat skin prepared with unbuffered saline and ex-
cised at one hour and 24 hours post burn and acutely burned rat sera
with animals sacrificed immediately after burning, at five, ten, and
30 minutes, and at 12 and 24 hours post burn. Normal skin extract
and normal serum were employed as controls.

Five different unboosted convalescent sera were tested with all of
the different presumptive burn toxin sources. None gave precipita-
tion lines on the Ouchterlony plates although some clouding was ob-
served.

With boosted antisera a precipitation line was produced with rat
sera collected up to ten minutes following the burn but not thereafter.
This distinct line on the Ouchterlony plate, however, was demon-
strated in only one out of five boosted convalescent sera which were
tested; it might be compared with the non-specific reactions which
have been described recently by Berenbaum and co-workers.
Considering the possibility that the antigenic source might have been

one or more products of blood hemolyzed following thermal injury, e.g., hemolyzed red blood cells, destroyed leucocytes or lysed platelets, tests were made with controls of plain and heated normal unhemolyzed and hemolyzed blood, hemolyzed red cells, and plasma. All of these were negative. Lysed white cells and platelets and necrotic cells were not studied in view of the inconsistent results obtained. At any rate, it was concluded that should the precipitation line represent a true burn antigen it is apparently cell-fixed or at least non-circulating very shortly after the burn.

A further study was carried out using lymph as the presumptive source of burn antigen. Convalescent sera were collected from two dogs which after a complete recovery from a 30% surface area burn had received a second burn. Pre-and-post-burn lymph were collected after cannulation of the left thoracic duct in five dogs and tested against two different convalescent sera. Neither lymph obtained immediately post burn nor that collected in a period of two hours after the burn formed a precipitation line.

Studies made with heterologous antisera showed no significant differences between normal rat serum and serum taken at various intervals post burn. With skin extracts, more lines resolved from

burned tissue preparations than from normal controls.

For immunoelectrophoresis studies the method of Graber-Williams was employed with the modifications described by Wienne. In particular, a 1.5% Noble Agar, buffered to a pH of 8.2 was prepared. A current of 25 milliamps. at 130 volts applied over 20 minutes was sufficient for good resolution of antigen on a 3 x 1 inch slide. Essentially the same findings were noted as in the Ouchterlony plates although precipitation lines were somewhat less well defined.

As a final study, the Schultz-Dale test, which depends upon sensitization of smooth muscle with antigen and perfusion with antibody-containing agents to produce a contraction which may be recorded on a drum tracing, was undertaken with the assistance of L. J. Olson,[37] utilizing his apparatus and methodology, and employing both ileum and uterus of the guinea pig sensitized with normal rat serum. In pilot studies two positive reactions were recorded from the ileum in three animals sensitized with acute rat burn serum taken six minutes after injury. Thereafter the following experiment was conducted:

Experiment V

Series A. Female guinea pigs were sensitized with rat serum containing presumptive burn antigen. The serum was collected at various intervals after a burn of 40% weight immersion with water of 90° for 20 seconds. The serum was diluted with saline (1:5) and three doses of 0.5 cc. were injected intraperitoneally at three day intervals. The Schultz-Dale experiment was performed only 15 days after the last injection in an effort to achieve a high antibody titer.

Using the ileum and the uterus of the sensitized guinea pigs, a contraction of the smooth muscle with the rat burn serum, after repeated desensitization of the test organ with normal rat serum should provide evidence of specific antigen-antibody reaction.

Five guinea pigs were sensitized in each group with serum as follows:

Group 1. Rat serum collected six minutes post burn.

Group 2. Rat serum collected 30 minutes post burn.

Group 3. Rat serum collected 24 hours post burn.

All guinea pigs were successfully sensitized against rat sera, and no deaths occurred. In no instance could a specific burn-antigen-antibody response be demonstrated.

Series B. In a second series, lymph was collected from the left thoracic duct of five dogs before and immediately following a 30% surface area burn in hot water of 90° for 20 seconds. Four guinea pigs were sensitised with the lymph of each of the experimental animals, using the same technique as in Series A. No deaths occurred. The Schultz-Dale tests were again performed 15 days after the last lymph injection. In no instance was a further muscle contraction demonstrable with burn lymph after complete desensitization with pre-burn lymph of the same dog.

These studies demonstrated the technical difficulties of Schultz-Dale tests and gave further evidence of laboratory inconsistencies.

CONCLUSION

The consensus at present by the majority of workers in this country with respect to convalescent burn serum as summarized recently by Pennell [22] is that while proof is lacking for the existence of a specific burn toxin of antigenic nature and of corresponding specific burn antibodies, "there are indications .... that specific antimicrobial antibodies may be present in the blood of organisms which have recovered from infected burn wounds. It is within the realm of possibility that beneficial effects might result from transfusion of blood,

plasma, or serum from a donor possessing antibodies which react
specifically against micro-organisms infecting a recently burned
organism. "

In spite of the discouraging results obtained to date in our laboratory
and by other workers in the field, there is considerable impetus toward
continuing laboratory investigation of the problem employing both
in vitro and in vivo experimentation in suitable animals, and these
are being pursued in addition to studies to evaluate the qualitative
factors in convalescent burn serum which would relate to neutraliza-
tion of bacterial toxins. Meanwhile the results of clinical trial are
either not available in sufficient detail for conclusions to be drawn
as to the efficacy of convalescent serum therapy or are being conduc-
ted without uniform protocols and in insufficient numbers for assess-
ment of its value and the concomitant risks involved, if any exist.
Little mention has been made of the logistic difficulties of collection
of serum from convalescent burn-patients, although some efforts have
been made by national organisations to assist physicians in obtaining
donors for clinical trial. This problem has been emphasized by
Russian and Czech workers.

It is apparent from experimental studies that antigen-antibody

phenomena do occur following thermal trauma but that attempts to reproduce results have been technically difficult with often inconsistent findings, even in the same laboratories. Furthermore, the significance of such reactions is not clear. If they are related to toxic symptoms, statistically valid data are not as yet available for confirmation of a specific burn toxin-antitoxin hypothesis.

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A toxin has been isolated directly from diffusates of burned
skin of rats. This toxin, which is lethal to rats and mice,
is dialyzable, heat-stable, and only partially precipitated
by 80 per cent ethanol. Tests indicated that it appears to
contain peptides, polynucleotides, hexoses, pentoses,
histamine, bradykinin, and adenyl compounds. There is
a difference between the lethal effects produced in mice
by using the crude toxin and its dialysate. The dialysate
separated from the particulate matter has less effect.

Shortly after injury, cytotoxins and a substance which
precipitates in the presence of convalescent serum appear
in the serum of burned mice, rats, and humans. The
cytotoxins are demonstrated by lysis of the subject's
erythrocytes in the presence of the subject's serum and
added (guinea pig) complement. With healing, precipitins
and hemolysins appear.

Injection into rabbits of the toxin obtained from diffusates
of the skin of burned rats plus Freund's adjuvant produces
in the serum of the rabbit precipitins to the toxin and
hemagglutinins and hemolysins to red cells of recently

burned rats. In mice, the serum of such rabbits exerts a protective effect against the toxin obtained from burned skin diffusates of rats.

Toxins and antitoxins were demonstrated in the serum of pathogen-free and normal rats àfter burn injury of 20 per cent of the body surface, but the antitoxins appeared only rarely and were present in very low titers in the pathogen-free animals.

A substance was found in the serum of recently burned children which inhibited the growth of HeLa cells in tissue culture in 17 of 20 specimens. Anti-inhibitory substances were found to occur in normal serum and in serum taken from subjects who had recovered from burns. These substances were titrated in tissue culture. Serum and gamma globulin from donors who had recently recovered from burns were found to have higher than normal anti-inhibitory titers. Examination of serum of recently burned children by modified electrophoretic techniques demonstrated a reduction of the $alpha_2$ peak and an abnormal component. This new moiety migrated

more rapidly than albumin and was not detected in the
serum of normal children.

Rosenthal has concluded that there is a toxin which is antigenic
and under certain circumstances an antitoxin may be developed
both naturally and artificially against the toxin.

Malm, [29] working at the Medical College of Virginia, reported a
10-day mortality of 21 per cent among rats which had received a
burn of 50 per cent of skin surface and which were given sterile
serum from rats burned seven to eight weeks previously. This
compared to a 10-day mortality of 95 to 100 per cent when rats
identically burned were treated with dextran-saline, sterile serum
from unburned rats, or sterile serum from rats from which about
30 per cent of the dorsal and flank skin had been surgically excised
seven to eight weeks previously. However, the rats receiving the
sterile serum from burned rats also died 13 to 16 days following
the burn. Malm[29] repeated these experiments later at the Walter
Reed Army Institute of Research using pathogen-free rats. In
these initial studies, the animals tolerated the burns so well
that distinction could not be made between immunotransfusion and
other forms of treatment. On the basis of these experiments,

Malm postulated that any protective effect which convalescent burn
serum might have is largely or exclusively due to bacteriocidal
or bacteriostatic effects.

Very recently, Malm and Slawikowski[3] prepared a monograph
describing much more extensive experiments conducted at WRAIR.
Appendix A is a summary of the results of this work. The studies
were undertaken to "re-evaluate the effect of the burn convalescent
serum with due emphasis on details of methodology and on the
statistical validity of the data obtained." It was postulated that
unless conclusive evidence could be presented to demonstrate that
the use of such sera could reduce mortality, exhaustive studies
necessary for the complete evaluation of the problem could hardly
be justified.

In the most recent studies, about 2, 000 adult male pathogen-free
rats of the Walter Reed Wistar strain were used. Experimental
conditions were controlled meticulously and studies were done on
a double-blind basis.

On the basis of these studies, the authors have concluded that:
"The burn convalescent serum was shown to lower the mortality
following severe, but not following moderate, burns. This observa-

tion is consistent with but not as striking as previously reported. Other sera did not reduce mortality.

"Chloromycetin alone lowered mortality following severe burns; burn convalescent serum or normal serum given in conjunction with chloromycetin did not offer additional protection.

"The results of these studies are compatible with the burn toxemia previously postulated, although it probably represents only a minor factor in the mortality after severe burns.

"The importance of meticulous controls and of careful evaluation of experimental data was clearly demonstrated."

In the past, however, many other investigators including Underhill and Kapsinow, [30, 31] Harrison and Blalock, [32] Harkins, et al., [2, 33] and Krauel and Payne[34] have been unable to demonstrate the presence of toxins in burned tissue fluids and extracts, and consequently the presence of antitoxins.

During recent years, several other investigators in this country have attempted to duplicate studies from which data have been derived in support of the burn toxin-antitoxin concept. A representative number of these were asked to summarize the results of

their recent work and these summaries are appended to this report, as follows:

Malm and Slawikowsky - Appendix A

Newton and Fujii - Appendix B

Sell - Appendix C

Graber - Appendix D

T. Blocker - Appendix E

Sanford Rosenthal - Appendix F

Miller - Appendix G

The information contained in Appendices B through G and additional data presented at the meeting in August are summarized in very brief form in the following paragraphs.

In an effort to demonstrate the existence of the proposed burn toxin in in-vivo animal preparations, Graber, T. Blocker, and Sell have confirmed the observation that injection of crude diffusates or extracts of burned skin when given to rats in large doses by various routes of administration causes death in a significant percentage of the animals within a short period of time. However, if the diffusates or extracts are carefully cleared of particulate matter before injection, no such lethal effects are observed.

In attempts to evoke anaphylactic reactions in guinea pigs as described by Feodorov, extracts of burned skin and normal skin, and serum from burned dogs, rats, rabbits, and guinea pigs have been injected into suitably prepared guinea pigs by several routes of administration by Newton, Fujii, T. Blocker, and Sell. Invariably, when test materials used as antigens were carefully cleared of particulate matter prior to injection, it has not been possible to induce sensitization and consequently to produce anaphylactic reactions. However, T. Blocker and Sell have observed anaphylaxis in guinea pigs when crude unfiltered extracts of burned skin served as the antigen.

T. Blocker has been unable to demonstrate a significant difference in survival at the end of one week after burning between groups of rats treated with convalescent rat burn serum and untreated controls. He questions the use of ultimate survival after burning as an index for evaluating the properties of convalescent burn serum in animals because of the influence of the many other factors on the outcome of the traumatic experience. In Appendix A, Malm reports that use in rats of "open-wound convalescent burn serum" and "repeated burn serum" increases the mortality during the first 48 hours after burning. In noting this finding, Carl A. Moyer of

Washington University calls attention to the fact that excision of
an intact burn eschar converts a closed wound to an open one. In
the experimental animal, this procedure introduces an entirely
new set of experimental conditions which are reflected in observa-
ble changes in the animal. In clinical practice, burns are usually
converted from closed to open wounds during the course of
treatment. Therefore, interpretation of data obtained from
studies of animals which are allowed to retain the burn eschar
must be made with great caution. T. Blocker and Sell have also
observed that animals which were burned repeatedly were notice-
ably more affected by burns subsequent to the first. In many cases,
an animal died from a second burn after having survived and
apparently completely recovered from a much more severe
previous experience. John Howard of Hahnemann Medical School
reported on a study currently in progress in which humans are given
a small standardized burn on one forearm, which is repeated one
month later on the other forearm. He noted that in 200 such
experiments no differences have been observed between the indivi-
dual traumatic experiences.

Sell and T. Blocker stressed the influence of experimental tech-
niques on the outcome of experiments on animals. For example,

if 1.5 ml. of serum is rapidly injected intravenously into a normal
adult rat, the animal dies in a significant number of instances.
If the injection is given over a period of several minutes, this
effect is not encountered. Malm[3] has also called attention to
several other variables in experimental techniques, including
control of temperature, humidity and fluid intake, and estimations
of surface area burned, which exert a profound influence on the
results of studies on burned rats. In this respect, it is to be
noted that on repeating the work of Simonart concerning the toxicity
of polypeptides obtained from edema fluid of burned animals
Allgower,[35] using sterile materials, failed to substantiate these
findings.

In addition to the in-vivo experiments on animals, a considerable
number and variety of in-vitro studies are being conducted.

Miller at NAMRU #4 has continued studies on burn serum using
electrophoretic techniques. Recently, he has completed a double-
blind study of 64 sera from burned patients. These sera were
from the same specimens used by Sell in tissue culture studies
reported in Appendix C. Miller has been unable to identify the
previously reported pre-albumin moiety. Using serum of dogs

which were burned 24 hours previously, he has demonstrated a reduction of the albumin peak and a new component in the B_2 globulin region. T. Blocker has studied chyle obtained from the catheterized thoracic duct of dogs before and following the infliction of a deep scald burn of a hind leg. He found that within one hour after burning, the chyle became dark red. At two and one-half hours, the electrophoretic pattern demonstrated a decreased albumin peak and a marked elevation in the B-globulin area which then decreased to almost pre-burn levels by 12 hours after injury. His opinion is that this material is a denatured hemoglobin. He has also injected radio-iodinated serum albumin into dogs and noted a concentration of 50 per cent of the amount given in the tissues surrounding the burned area within a few hours. The albumin in only slightly decreased amount was still present in the burned area 10 days after administration. J. F. Saunders of the Office of Naval Research, reporting on preliminary work of Nicholas G. Georgiade at Duke University, noted changes in the electrophoretic pattern of serum obtained two to seven days following burning. The possibility that there is a marked decrease in the free amino acid content of such sera is being investigated.

In a small series of experiments, Sell has been unable to duplicate

the results reported by Sol R. Rosenthal concerning the effect of
acute burn serum and convalescent burn serum on smooth muscles
of guinea pigs in muscle bath preparations.

Graber and T. Blocker have had inconclusive and inconsistent
results in studies employing serological techniques in attempts to
demonstrate burn toxins and antitoxins. T. Blocker observed an
increased hemolytic effect of noninactivated serum of burned rats
on washed erythrocytes of rabbits. However, he noted that
serum from normal rats tends to lyse erythrocytes of normal
rabbits, and thermal trauma to rats seems to enhance this effect.
Graber injected crude, unfiltered diffusate of burned skin plus
Freund's complete adjunct into rabbits. He then tested serum
from these animals against unmodified sheep cells sensitized with
crude diffusate of burned skin, and noted a low order of hemagglu-
tination for which he offers no explanation. In a single experiment
using serum from an acutely burned patient and from another who
had recovered from burns, Graber was unable to demonstrate
the presence of hemolysins or hemagglutinins as reported by Sol
R. Rosenthal.

Using immunoelectrophoretic techniques, Sell and T. Blocker

have demonstrated the presence of a new antigen in the serum and burned skin of dogs and rats after burning. In these preparations a diffuse area of precipitation was produced. T. Blocker has also shown that precipitation lines may be demonstrated by using serum of twice-burned rats, but these lines are not considered to be specific. Using Ouchterlony plates, Sell has been unable to demonstrate precipitation bands between rabbit serum collected within 24 hours after burning and serum collected two weeks after healing. However, T. Blocker, using rat serum obtained within 10 minutes after burning or extracts of burned skin taken 24 hours after burning, has demonstrated a cloud of precipitation by the Ouchterlony technique. Again, when the tests were repeated using serum from twice-burned rats, distinct lines of precipitation were produced. At present, this finding is interpreted simply as an indication of an antigen-antibody reaction.

Sell and Graber have also made extensive studies in tissue culture preparations employing a large variety of cell strains, including HeLa, Lilly embryo skin and muscle, Henle's lung, Earle's adult skin, monkey kidney, mouse liver, primary guinea pig kidney and cloned monkey kidney on chemically defined media. Crude extracts of either burned or normal skin have been shown

to have cytotoxic effects which, however, are not observed if
the extract is cleared of particulate matter. In double-blind
studies, no cytotoxicity was demonstrated in acute burn serum
if the specimen was immediately removed from its clot and
maintained in frozen state. If, however, the serum is allowed
to remain in contact with its clot under certain circumstances,
the serum does become cytotoxic. This same result may be
obtained by using normal type AB serum, which prior to storage
with its original clot was shown to be noncytotoxic.

Miller has several reservations about the results of previous
studies of acute and convalescent burn sera done at NAMRU #4
by tissue culture techniques. He points out that under the
emergency conditions in which these studies were conducted it
was not possible to obtain data which could be adequately assessed.
The data collected were largely subjective impressions and were
not obtained on a double- or even a single-blind basis. Adequate
bacteriological data were not derived and because of the many
variables in the treatment given, the significance of any one form
of therapy could not be determined.

Finally, Edsall, [38] in commenting on the reported lack of

specificity of the proposed burn toxin, raises the point that there may be in fact a specificity arising from bacterial antigens produced by organisms infecting the burns which are common in all the various species of animals studied.

III. THE STATUS OF CURRENT INFORMATION
FROM CLINICAL INVESTIGATIONS

Very limited clinical experience with use of immunotransfusion in burns has been acquired thus far in this country. Rosenthal[24] has reported improvement in six burned children each of whom received immunotherapy consisting of repeated infusions of 250 ml. of plasma or 500 ml. of blood obtained from individuals who had recovered from extensive burns. The infusions were not begun until the fourteenth day following the burn and were given as part of a very intensive therapeutic regimen including use of antibiotics. Reported beneficial results include reduction of pain, fever, edema, irritability, and albuminuria. In addition, Rosenthal has received a few reports of seemingly beneficial clinical effects from physicians who have used plasma and serum from individuals who recovered from burns. Data from these experiences are not available.

Reports of the use of immunotransfusion on the European Continent are more numerous. Pennell[36] reports that transfusions of convalescent burn plasma have been made at the Army Center in Clamart, France. Feodorov has made repeated reference to use of immunotransfusion in Russia, but no detailed written account was available in this country until 1961. Recently, Pushkar[37] has reported on a series of 940 burn cases in which immunotherapy was used in 114 patients in conjunction with the usual comprehensive method of treatment.

In this series, the donor blood was taken from patients who had recovered from a burn of at least 7 per cent of skin surface and was drawn within at least six months after recovery. Two hundred and eleven transfusions of this blood, usually in the amount of 250 ml., were administered to patients with burns of 10 to 96 per cent of skin surface. A marked improvement in the clinical picture of toxemia resulted, characterized by decreases in dyspeptic disorders, leukocytosis, and fever. Renal function returned to normal. Healing of the wound was more rapid, skin grafting could be performed earlier, and the septic stage was favorably affected. Of the 114 cases treated, 111 survived the phase of toxemia. Apparently 12 patients in the series died

ultimately. On the basis of statistical analysis, it was concluded

that immunotherapy is effective only in cases suffering burns of

more than 10 per cent of skin surface. Additional information

and comparative analysis of the data reported by Pushkar are

contained in Appendix H.

Edsall, [38] who visited the laboratory of Feodorov in August, 1961,

was told by Feodorov that: "In the treatment of human patients,

the plasma should be taken from a donor who suffered burns

at least 30 to 40 days previously; preferably serum (or plasma)

should be used that is taken a year or so after the donor was burned

.... not all the sera of burned patients develop antitoxic properties.

In treating patients, give the serum as soon as possible after the

burn has occurred."

Pavkova, Dobrkovsky, and colleagues[11] recommend administration

of serum from healed burn cases (in which tha antibody titer by

collodion particle agglutination is at least 1:64) to the newly

burned patient as soon after injury as possible. For an adult,

a dose of 100 ml. per day for four days is suggested. This group

has studied and reported on 10 such patients who, in addition,

received therapy usually prescribed in that area for treatment of

burns and has concluded that: "Further careful and assiduous investigations will be necessary, of course, but we do believe that immunotherapy will favorably supplement the usual up-to-date treatment of the seriously burned." At present, this group is treating burned children routinely with such immunotransfusions. The treatment is also used for adults, but because of limitations in supply of serum, is restricted to those who are severely but not hopelessly injured.

No reports are available concerning the clinical use of immunotherapy in England. However, there is some sentiment[39] in that country for a controlled clinical trial on carefully selected cases.

On the other hand, Sanford M. Rosenthal, in summarizing his experience with a large number of well-controlled studies on burn patients in the "Peru Project," is of the opinion that little effect against burn shock may be anticipated from use of convalescent serum in adults. In his hands, use of large doses of saline solutions has produced a zero mortality from shock in over 100 cases of burns of up to 50 per cent body surface. He feels that this militates against a bacterial factor or toxin as a cause of death

during the shock phase but admits the possibility that the anti-

bacterial antibody content of convalescent serum may have some

protective value against delayed deaths from burns in adults.

In young children there is good evidence that bacterial invasions

by Pseudomonas and Staphylococcus may begin during the shock

period and contribute to early as well as late mortality. While

plasma and large doses of gamma globulin have a pronounced

effect in children, it is possible that convalescent serum might

be more active. A trial is being conducted in Lima on this score.

IV. SUMMARY AND CONCLUSIONS

It has been proposed that a significant injury of the skin by thermal

trauma results in production of a substance in the traumatized

tissues which is both toxic and antigenic. This substance pervades

the burned organism and causes a toxemia. In favorable instances,

the toxic substance stimulates formation of an auto-anti-toxin or

auto-antibody which counteracts the toxemia. In addition, injec-

tion of the toxin into another organism under certain circumstances

stimulates the formation of an antitoxin in that organism. The

antitoxin may therefore be produced naturally or artificially.

Finally, neither the toxin nor antitoxin is species-specific.

On the basis of this thesis, a method of therapy, immunotrans-
fusion for burns, has been advanced. Blood is drawn from an
individual who has recovered from significant burns and either
the whole blood, plasma, or serum is infused into the recently
burned patient on the presumption that the procedure favorably
influences the toxemia and possibly the final outcome of the
course of illness.

For the purpose of this study, a large amount of data and infor-
mation on this subject obtained from all available sources was
reviewed and analyzed from the following three viewpoints:

First, the validity of evidence in support of the existence of a
biologically active autotoxin in the burned organism.
Second, the validity of evidence in support of the occurrence
of antigen-antibody phenomena in the burned organism.
Third, the effectiveness of the use of immunotransfusion
following burning in man and experimental animals.

It is concluded that:

    A. There is no statistically acceptable evidence to
suggest support for the burn toxin-antitoxin concept.

    B. Some evidence exists to suggest that antigen-antibody

phenomena do occur in the living organism following burning, but the biological significance of these phenomena is unclear. However, at this stage of development, it seems permissible to conjecture that if a biologically active antigen resulting from burn injury does exert a deleterious effect, it is a morbid rather than a mortal one.

C. The data available on the use of immunotransfusion in man and experimental animals are inconclusive. The scope of the studies performed to date on this problem is limited, and many of these studies are completely negative. No statistically acceptable evidence has been acquired to demonstrate that the clinical use of convalescent burn serum, blood, or plasma-surpasses in value other methods of treatment to a 5 per cent confidence level. The preponderance of evidence from animal experiments fails to support the thesis that such transfusions have any specific beneficial effect against an autotoxin or auto-antigen. There are indications, however, that specific anti-microbial antibodies may be present in the blood of organisms which have recovered from infected burn wounds. It is within the realm of possibility that beneficial effects might result from transfusion of blood, plasma, or serum from a donor possessing

antibodies which react specifically against microorganisms infecting a recently burned organism.

Additionally, with respect to further investigation of the clinical use of immunotransfusion, there is no basis upon which to determine the dosage level for a broad-scale clinical study without positive pilot studies in animals. To set up a clinical study with the objective of detecting a 20 per cent or greater benefit with a confidence level of 5 per cent in a population as heterogeneous as clinical burn populations would require a very large series of miscellaneous burns or a substantial series of selected burns in order to include the proper controls. Assuming a requirement of 2 liters of burn plasma per case, half of a series of 1, 000 burns would require 500 ml. blood donations from each of 2, 000 convalescent burn patients. Because under existing operational standards blood banks do not accept donors within six months of the receipt of a transfusion, separate donor programs might have to be developed.

     D. The causes of morbidity and mortality, particularly in the more extensive thermal burns, and the definition of optimal methods of treatment present significant problems requiring further study.

V. SUGGESTED COURSES OF ACTION

These suggestions are grouped under two headings - first, laboratory
investigations which include both in-vivo and in-vitro experimentation,
and secondly, clinical investigation. In addition, the individuals
responsible for conducting this review agreed unanimously on the
desirability of repeating in this report the admonition that a reaction
observed in an in-vitro preparation may not be the same as, nor in
any way related to, that which occurs in the living organism. Only
through carefully conducted confirmatory in-vivo studies can such
observations be established as biological facts.

A.   Laboratory Investigation

1. In-Vivo Animal Studies

Further investigation should be conducted using animals,
especially large animals, subjected to standardized burns under the
very best laboratory conditions of animal care and with meticulous
control of temperature, humidity, and other such variables. In
these studies, it is of paramount importance to observe strict micro-
biological controls.

Protocols of such studies, as applicable, should be designed to
simulate as nearly as possible the standard regimens used in clinical

practice.

In addition, studies using all appropriate methods, including immuno-logic techniques, should be continued in an effort to demonstrate and characterize toxins and autoantigens, and to adequately evaluate the effectiveness of immunotransfusion in animals.

The findings of the above studies should be reviewed in relation to the current clinical care of the severely burned patient.

2. Basic

a. Biochemical Techniques

There appears to be no evidence to suggest the advisability of extensive biochemical study of possible changes in cellular metabo-lism or in plasma proteins following burning. On the other hand, interesting leads have been reported in both of these areas. It appears unlikely that additional information concerning these abnormalities from the biochemical viewpoint would bear directly on the question of use of immunotransfusion in burns. However, in the broader context, it is important to pursue studies in this area to establish additional scientific facts.

b. Tissue Culture Systems

In general, tissue culture techniques previously used in studies concerning the proposed toxin-antitoxin phenomenon in burns have been inadequate. Today, a larger spectrum of cell types and strains can be grown in quantities which were relatively impossible a few years ago. A substantial number of cell types and strains can be induced to proliferate rapidly under more accurately controlled experimental conditions and in solutions containing no added protein. Refinements have been made recently in collateral techniques which make tissue culture of greater usefulness in studies involving retardation or acceleration of cellular proliferation, migration of cells, and changes of specialized cellular functions such as secretory or enzymatic activities. These newer tissue culture and refined collateral techniques could be employed with definite value in further studies of this problem.

c. Immunological Techniques

In addition to studies designed to evaluate the therapeutic effectiveness of immunotransfusion in burned animals, it would be desirable to investigate the presence of antigens and antibodies characteristic of the burned animal. In these studies, efforts should be directed toward achieving the best antibody response and toward concentrating antiserum to increase the sensitivity. In this connection,

successive burning as a means of achieving a more potent serum should

be explored. Efforts should also be continued to determine whether

or not convalescent burn plasma or serum in itself is harmful.

However, apart from considerations of any protective effect of convales-

cent burn plasma or serum or the existence of a burn toxin, there

may be changes in the blood or body fluids of a burned organism

which may be susceptible to demonstration by immunological and

immunochemical methods. The significance of such changes may be

fundamental to understanding of the patho-physiological changes

occurring in the burned organism. However, in the absence of any

demonstrable protective effect of immunotransfusion in the burned

organism, changes observed in the laboratory by immunological

methods do not constitute a basis for the clinical use of convalescent

plasma or serum.

B. Clinical Investigation

Neither large-scale clinical studies nor collection and stock-

piling of convalescent burn plasma or serum on a nationwide basis

and its distribution on request to physicians can be recommended at

the present time. Such actions should not be considered unless

further studies in animals are completed which yield clearly affirm-

ative results. Indeed, there may be inherent dangers in the use of convalescent plasma or serum which are not fully recognized at present.

Pilot clinical studies by individual investigators or groups of cooperating investigators with fresh approaches to various controllable facets of this problem will undoubtedly be continued, and support for such projects should be afforded on the merits of the individual proposals. Such pilot studies when undertaken should be on a double-blind basis using "normal" plasma or serum in alternate cases. It would also be important in such studies to record for future evaluation the age, blood type, date on which the donor's burn was sustained, the area, depth, location of his burn, results of microbiological cultures at intervals during the healing period, amount and types of colloid and blood received, and the time required for healing. All of these factors might have significance in characterizing a particular donor's plasma or serum. Finally, the time of death or the incidence of hepatitis should be recorded for the recipient.

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APPENDIX A

Summary from Monograph

Drs. O. J. Malm and G. J. M. Slawikowski
Office of the Surgeon General
Department of the Army
1961

Severe cutaneous contact burns were studied in adult male rats to evaluate the relative effect of various types of sera on the mortality following these burns.

Pertinent literature was reviewed, with special attention to burn toxemia and its treatment with convalescent sera.

Careful attention was given to experimental design and conditions, with emphasis on randomization and the double-blind approach. A hot plate burning technique was developed which yielded reproducible results in any given experimental series when similar treatment was applied to different experimental groups. Deaths in the shock phase (the first 48 hours) were minimized and equalized by withholding food and water for the first 24 hours, and giving subcutaneous injections of physiological saline in the dosage of 14 ml. per 100 gm. of body weight in divided doses (immediately after, and again three hours after the burn).

The therapeutic sera were collected under sterile precautions and

tested for bacterial contamination both at the time of bleeding and at the time of pooling. They were given in divided doses, 1.4 ml. per 100 gm. of body weight during the first week, corresponding to nearly 1 liter of serum in a 70 kg. man. Several types of sera were evaluated:

1. Normal serum from unburned rats;

2. Serum from unburned rats given control injections of Freund's adjuvant;

3. Serum from rats burned repeatedly, five times, five days apart (serum collected 10 days after the last of five burns);

4. Two-month, three-month, and five-month burn convalescent sera;

5. Two-month and five-month open wound convalescent sera;

6. Three-month test-burn survivor serum;

7. Three-month fracture convalescent serum; and

8. The normal, or three-month burn convalescent serum, supplemented with chloromycetin.

The two-, three-, and five-month burn convalescent sera caused a consistent, though small, decrease in mortality following severe burns; this protective effect was not seen following burns involving less than 50 per cent of the skin area. A significant protective effect was not seen with any of the other sera tested. The open-wound convalescent serum and the repeated-burn serum increased the mortality during the first 48 hours after burns.

Chloromycetin reduced mortality following severe burns; chloromycetin supplemented with the three-month burn convalescent serum or normal serum was not more effective than chloromycetin given with saline alone.

Ancillary studies revealed that burned skin harbored Proteus vulgaris, E. coli, and Staph. aureus. Chloromycetin reduced the population of Proteus in situ; no antibacterial activity against the Proteus was found in any of the sera tested.

Analysis of the body weight of burned animals revealed that the moribund animals retained much more fluid during the first 48 hours after burns than did the survivors; this may be indicative of greater circulatory and/or renal embarrassment in the former. It was noted that only groups treated with the burn convalescent serum did not reveal this difference between survivors and rats dying within 10 to 60 days. This may indicate a protective effect of the burn convalescent serum on the circulatory and/or the renal system.

The body temperatures of rats destined to die within 10 days were considerably lower than those of rats surviving 10 days. This indicated primarily a slower recovery from the shock phase.

Hematocrit studies revealed severe hemoconcentration early after

the burns, followed by a gradual return to normality after 12 hours.
Since water was withheld for 24 hours after burns, these changes
(which occurred in the presence of saline given by subcutaneous in-
jection, 14 ml. per 100 gm. of body weight in divided doses immedi-
ately and three hours after burns) represented shifts in internal body
fluids not influenced by variation in intake.

There was some indication that the burn convalescent serum may have
exerted its protective effect even within the first 48 hours, although
at the end of 48 hours this effect was not reflected in the mortality
figures. During the next 72 hours a consistent and statistically signi-
ficant difference in mortality occurred between groups treated with the
burn convalescent serum and the normal serum. Between five days
and fourteen days after burns there was little rebound mortality.
Cumulative mortality figures therefore continued to show statistically
significant differences between rats treated with the burn convalescent
serum and those treated with the normal serum.

The accuracy and reproducibility of the burning technique was confirmed
by the measurement of skin areas of burned and unburned rats using
a new technique based on the relationship of the aluminum foil weight
to it.

The results are discussed in the light of previous reports. The results to date are compatible with the view that a burn toxin is responsible for the mortality between 48 and 120 hours after severe burns; however, this toxin is probably a minor, rather than the most important etiological factor in the death after severe burns -- it may be the "straw that breaks the camel's back." The clinical significance of these findings is discussed.

APPENDIX B
Attempts to Reproduce the Feodorov Phenomenon - Burn Antigens
William T. Newton, M. D., and Koichi Fujii, M. D.
(Data obtained through the courtesy of Dr. Carl A. Moyer)
August, 1961

Part I:

Number of Guinea Pigs	IMMUNIZATION			
	Burn Antigen[1]	Amt. (ml.)	Route[2]	Time[3] (days)
5	Rabbit serum[4]	1.0	F. P.	26
9	Guinea pig serum[4]	1.0	F. P.	26
1	Guinea pig serum[4]	1.0	I. V.	26
6	Guinea pig blood,[5] then guinea pig burned.[6] Skin homogenate - 27 days later	1.0 0.8	F. P. S. C.	28
1	Guinea pig blood[5] Burned skin homogenate - 27 days later[6]	1.0 1.0	F. P. I. V.	28
4	Guinea pig blood skin mixture[8] Burned skin homogenate next day	1.0	S. C.	21
5	Guinea pig blood skin mixture[8] Burned skin homogenate next day	1.0 1.0	F. P. S. C.	21

APPENDIX B
(Continued)

Part II:

(# of G. Pigs)	INTRAVENOUS CHALLENGE	
	Burn Antigen[1]	Anaphylaxis
5	Guinea pig serum[4]	No symptoms
9	Guinea pig serum[4]	No symptoms
1	Guinea pig serum[4]	No symptoms
6		
	Guinea pig plasma[8]	No symptoms
1		
	Guinea pig plasma[7]	No symptoms
4		
5		
	Guinea pig skin extract[9]	No symptoms

APPENDIX B
(Continued)

(1) All antigens obtained from animals burned 48 hours previously by applying flaming alcohol on cotton wads to the clipped skin of the back for 60 seconds. The dry third degree burn eschars were from 20% to 30% of body surface. One animal died, the only one that developed a "wet eschar." All antigens were kept iced until used.

(2) F, P., footpads; S. C., subcutaneous; I. V., intravenous

(3) Interval between last immunizing injection and challenge

(4) Serum from blood allowed to clot overnight, clarified by centrifugation at 10,000 x g for 20 minutes.

(5) Blood obtained by cardiac puncture with Ethylene diamine tetracetic acid (EDTA) as anticoagulant.

(6) Burned skin was homogenized in ground glass homogenizers (approximately 10% W/V), in saline, filtered through 50 mesh stainless steel screen and used immediately.

(7) Plasma was separated from whole blood collected in EDTA tubes by centrifugation at 200 x g for 10 minutes.

(8) Equal parts of whole blood and 10% burned skin homogenate from burned guinea pigs.

(9) Supernatant fluid from centrifugation of 10% burned guinea pig skin homogenate at 5,000 x g for 15 minutes.

APPENDIX C

Summarization of Investigations Conducted at the
National Naval Medical Center, Bethesda, Maryland,
Concerning a Proposed Toxin and Antitoxin
in the Serum of Burned Patients

Kenneth W. Sell
August, 1961

I. Evaluation by Tissue Culture Techniques using HeLa Cells

Following the reports of Dr. Sol Roy Rosenthal that sera taken from

burned patients inhibited the outgrowth of newly planted HeLa cells in

test tube tissue cultures, attempts are being made to duplicate the

test situation as originally described. In our initial studies, more

than 218 sera were tested including over 160 controls. No cytotoxicity

of burn serum collected using sterile techniques and separated

immediately from its clot has been demonstrated. In these studies

the observable indices used as indications of cytotoxicity were failure

of the cells to become attached to glass and lack of outgrowth of newly

planted cells. A partial cytotoxicity was found in six of eight burn

sera allowed to remain in contact with the clot in the refrigerator

for a period of two weeks prior to testing.

In more recent studies, specimens of sera from patients were obtained

from Dr. Truman G. Blocker, Jr., of the University of Texas Medical

Center, Dr. Nicholas G. Georgiade of Duke University Medical

Center, and Dr. Robert F. Hagerty of the Medical College of South

Carolina. These specimens were classified as "acute" if drawn within
30 days after the patient was burned or "convalescent" if drawn 30
days or more after the patient had been burned. At the place of
procurement the serum and clot were separated and shipped in individual
containers to our laboratory by ordinary mail. The specimens were
received without knowledge on our part of the source of the material.
A file card system containing the information on each specimen was
set up and the cards kept sealed until our studies on the specimens were
completed. Upon arrival in our laboratory, the specimens were
stored in one of the following ways:

> all of the serum portion of the specimen was frozen.
> immediately and kept in frozen storage;

> a portion of the serum from a specimen was frozen
> and the remainder was reunited with the clot from
> which it had been separated at the place of procurement;

> the serum, in contact with its original clot, was stored
> in a refrigerator for two weeks, then removed, frozen,
> and stored in a frozen state;

> normal type AB serum, which previously had been shown
> to have no cytotoxic effect in HeLa cell tissue culture,
> was added to the clot of a specimen stored at refrigerator
> temperature for two weeks, then separated, frozen, and
> stored in the frozen state until tested.

The sera were studied by the routine HeLa cell test system in tissue culture test tubes. Results of the studies are tabulated as follows:

TEST SPECIMENS

Type of Specimen	Total	Cytotoxic	Noncytotoxic
1. Acute serum frozen immediately	19	0	19
2. Acute serum stored with clot	12	2	10
3. AB serum stored with clot from acute specimen	14	6	8
4. Convalescent serum frozen immediately	23	6	17
5. Convalescent serum stored with clot	17	11	6
6. AB serum stored with clot from convalescent specimen	15	12	3

CONTROLS

	Total	Cytotoxic	Noncytotoxic
7. Received from collaborators			
a. Frozen immediately	3	1	2
b. AB serum stored with clot	1	1	0
8. Own controls - frozen immediately	16	0	16

Discussion of Results

In view of results of previous studies, the results observed in experiments using acute serum stored in contact with the original clot were not surprising. No explanation is offered for these findings. However, in all the specimens prepared by storing noncytotoxic AB serum with clots from the! blood of acute burn cases, there was evidence of marked hemolysis. On the other hand, it is interesting to note that 6 of the 23 "convalescent" serum specimens which were frozen immediately were shown to be cytotoxic, as was one of the three controls received and frozen immediately. The significance of these findings is not understood. However, all six of the specimens of convalescent serum which were cytotoxic were obtained from one of the collaborators during a relatively short period, as was the single control specimen, which also proved to be cytotoxic.

These results, although of a preliminary nature, suggest that acute burn serum if separated from its clot and kept frozen until tested, is not cytotoxic in the routine HeLa cell tissue culture system. The results obtained from studies using convalescent serum are interesting but would seem to have little bearing on the subject of toxicity of acute burn serum.

II.   Evaluation by Tissue Culture Techniques using Other Cell Lines

Tests of the cytotoxic effects of burn serum have been performed in

several cell lines available in our laboratories, including monkey

kidney, cloned human skin, mouse liver stock, human stock, cloned

monkey on chemically defined media, and mouse liver. Of 16 acute

burn sera so tested, none showed any cytotoxic effects on these cell

strains.

III.   Evaluation by Tissue Culture Test with Primary Guinea Pig Tissues

Saline extracts of burned and normal skin of guinea pigs were tested

for cytotoxicity on already formed sheets of primary guinea pig kidney

tissue cultures. It was noted that when the crude extract of both the

burned and unburned skin were tested, evidence of cytotoxicity invariably

developed. However, if the saline extracts were centrifuged to remove

all particulate matter, neither the burned nor unburned skin extracts

had observable effect on the growth of the tissue culture cells.

IV.   Evaluation by Inhibition of Metabolism Cells in Tissue Culture

In these studies a bio-autographic system is being used. Growing cul-

tures of primary fresh kidney cells are overlaid with purified agar

and the test serum is allowed to diffuse through the agar. Inhibition

of cellular metabolism may be determined by evidence of localized

change in pH and failure of cells to remain attached to the walls of

the glass containers. To date, 10 burn serum specimens have been

tested by this technique and no evidence of cytotoxicity as indicated

by inhibition of cellular metabolism has been demonstrated. At

present, other specific metabolic functions of cells in tissue culture

are being evaluated as possible indicators for testing cytotoxicity

of burn serum.

V.   Evaluation by Guinea Pig Anaphylaxis Autoimmunity

Experiments have been made in which animals have been subjected

to standardized third degree burns* of 20 per cent of body area and

allowed to survive. At one to two weeks following healing they are

sacrificed and preparations of their smooth muscles are suspended

in a muscle bath for determination of anaphylactic sensitivity to

their own serum collected at varying periods following burn. Sera

has been taken from animals prior to burning for use as controls.

To date no autoimmunity has been demonstrated in animals using

acute sera collected at 24 and 48 hours after burning.

*A standardized burn machine has been constructed for use on small
laboratory animals. It consists of a ceramic plate infrared burner
with aluminum reflecting electronically controlled shutter and a water-
cooled contact plate. Animals may be burned to an area of 25.3
square centimeters with a very even distribution of heat which varies
from .83 - .87 calories per square cm. per second on a blackened
body surface. Using this apparatus, a third degree burn may be
accomplished in test animals with as little as a three-second
exposure.

VI.   Evaluation by Guinea Pig Anaphylaxis Heteroimmunity

Studies have also been done using guinea pigs sensitized to acute burn

sera from dogs.   The serum was obtained at varying intervals from

24 to 48 hours after burning.   Following immunization, the guinea pigs

were then desensitized with dog serum obtained prior to burning,

and again challenged with the acute dog burn serum to determine whether

additional antigens were present.   Such testing has to date failed to

demonstrate evidence of any new antigens at 24 and 48 hours postburn.

To determine the sensitivity of this test, the endotoxin of E. Coli was

mixed with dog serum in a quantity of 125 gamma of endotoxin to 1 cc.

of dog serum.   Guinea pigs immunized with this material were then

subjected to testing some weeks following immunization.   At this

time, marked sensitivity to the dog serum was demonstrated and

following desensitization, a residual sensitivity to endotoxin remained,

such that 12-125 gamma could be detected.   Thus, a small quantity

of a relatively weak antigen might not be demonstrated.   An attempt

is being made to revise the test system to increase sensitivity so as

to more adequately evaluate the presence of weak antigens in burn sera.

VII.   Evaluation by Guinea Pig Anaphylaxis using Saline Extract of

   Burn Skin

After careful centrifugation of extracts of burned skin, so that only

the soluble portion of the extract remained, we were unable to sensitize a series of five guinea pigs with material from either normal or burned skin. Therefore, in our hands no evaluation of new antigens in the burn skin extract can be made by this test system at this time. Further attempts to induce specific sensitization are being made.

VIII. Evaluation by Ouchterlony Agar Precipitin Studies

A series of five rabbits were subjected to standardized third degree burns of 20 per cent body area. Blood specimens were collected before and 24 hours after burning. The animals were allowed to survive and convalescent sera collected two weeks after healing of the burn lesions. These sera were then reacted together in agar plates following the technique of Ouchterlony. We have been unable to demonstrate any precipitin bands.

Similar studies were performed with five dogs using sera obtained 24 and 48 hours after burning. We failed to demonstrate antigens in the serum of burned dogs at these time periods.

IX. Evaluation by Immuno-electrophoresis

Studies utilizing antisera prepared in guinea pigs against burned dog sera taken 48 hours after burning have, in an initial two specimens, demonstrated a new antigen to be present which is not demonstrable

in the preburn nor 24 hours postburn specimens. An attempt is now

being made to determine whether this band is present in all 48-hour

burn specimens which are available from dogs and guinea pigs and,

if present, to determine the length of persistence of such new antigenic

bands. To date, this is the only laboratory evidence which we have

available indicating that a new antigen is found in the postburn serum

of animals or man. Of course, this antigen may or may not be a toxin.

APPENDIX D

Summary of Investigations Conducted at
U. S. Army Surgical Research Unit, Brooke Army Medical Center,
Concerning the Presence of a Toxic Antigen in the Blood
of the Recently Burned Organism and the Formation of Antitoxin
Antibodies Thereto and Studies on an Antibody Against Pseudomonas

Lt. Col. Charles D. Graber
31 July 1961

A. Burn Toxin

1. Toxicity

a. Animal - Anesthetized rats were burned on the dorsal
aspect, utilizing the pneumodermic technique of Rosenthal in which
a pouch of skin is raised by air. This pouch was perfused with water
at least 20 times and the eluate collected. The material obtained was
dried and rehydrated to give 110 mg. /ml. , and was given intravenously
in amounts of 0. 15 ml. Twelve rats and fifteen mice so treated were
unaffected. When the procedure was modified to allow burning of the
animal, followed by air insufflation to produce the pocket over the
burned area, nine of 18 animals given 100 mg. /ml. of the recovered
eluate died.

b. Tissue Cell Culture - Three tissue cell lines L, L, C,
H (Lilly, embryo, skin, and muscle), Henle's lung cell, and Earle's
adult skin were used in a Rose chamber to test toxicity of the diffu-

sate from the skin pouches on burned rat. When 15 mg./ml. of
diffusate was added to the chamber, dispersal of tissue culture
cells and some granulation but no degeneration were observed. However,
diffusate from nonburned animals produced similar results.

c. **Lysozyme Levels** - Eleven rats gave increases in lyso-
zyme levels from a preburn average of 2 mcgms./ml. to 6 mcgms./ml.
seven hours after suffering third-degree burn of 30 percent of body
surface.

d. **Shwartzman Sensitivity** - Dialyzed crude diffusate of
burned skin proved non-Swartzmanogenic for young rabbits given in-
jections of the material containing 100 mg/ml.

2. **Serology**

The crude burn diffusate used for toxicity studies was injected along
with Freund's complete adjuvant into rabbits to produce antibody
(antitoxin) following the recommendations of Sol Roy Rosenthal:
Specificity of Thermal and Radiation (Beta) "Toxins" of the Skin,
Rosenthal, S. R., Spurrier, W. A., and Trahan, H., Fed. Proc.
1960. Unmodified sheep red cells sensitized with the crude diffusate
were used to measure antibody produced in these animals. Table I
gives the results of this work.

Table 1

Hemagglutination Studies of Antibody Made in a Rabbit Against
Crude Burn Diffusate

	Rabbit Serum	Antigen	HA Titer 2 4 8 16 32 64 128
1.	unabsorbed	sheep RBC sensitized with crude burn diffusate	S S S S 4 ± -
2.	absorbed 3X with sheep RBC, 3X with normal diffusate	sheep RBC sensitized with crude burn diffusate	S 4 - - - - - -
3.	absorbed with sheep cells, normal diffusate and burn diffusate	sheep RBC sensitized with crude burn diffusate	- - - - - - - -

Although the burn diffusate absorbable antibody titer is not high, there
remains the expectation that a more concentrated antigen or a pro-
tracted schedule of immunization utilizing multiple injections with adju-
vant might result in higher titered antibody to burn diffusate. (This is
actually the only evidence from our laboratory that a "burn toxin" is
liberated in animals and antibody can be produced to it.) This material's
toxicity for rats and mice was questionable, as shown. The increases
in lysozyme levels may be damage per se to leukocytes from the burn.
This is an intriguing change not explainable by us at this time.

Attempts to demonstrate "burn toxin" in patients' sera in the acute

phase and antibody to this toxin in the convalescent phase was limited

to one series of experiments. The methods again were those of Sol

Roy Rosenthal as described in the J.A.M.A. 174:957-965, 1960.

In this paper he related how it was possible to demonstrate serolo-

gically hemolysins and cytolysins in the sera of children burned in

a Chicago fire. He also described antihemolysin and anticytolysin

antibody in the sera of children convalescing from burns suffered in

this fire.

In an attempt to reproduce this work, two patients were carefully

selected to conform to criteria stipulated by Dr. Rosenthal. However,

the patients in both cases were young adults, but the severity of

the burn and the bleeding intervals were the same as those reported

by Dr. Rosenthal. Our acutely burned patient was eight days post-

burn, (22 per cent total burn, 11 per cent of which was third-

degree); our convalescent burn patient was 70 days postburn at the

time of bleeding and had suffered a total burn of 51 per cent, 43

per cent of which was third degree. All lesions had healed in the

latter patient. Table 2 shows the results of the testing of the two

sera.

Table 2

Acute and Convalescent Burn Sera - Results of Serological Examination

1.	Acute burn RBC's	+	Acute burn serum (undil)	+	GPC'	→	no hemolysis
2.	Acute burn RBC's	+	Acute burn serum (1:5)	+	GPC'	→	no hemolysis
3.	Acute burn RBC's	+	Acute burn serum (undil)			→	no agglutination
4.	Acute burn RBC's	+	saline			→	no hemolysis
5.	Acute burn RBC's	+	GPC'			→	no hemolysis
6.	Acute burn RBC's	+	Burn conv. patient serum	+	GPC'	→	no hemolysis
7.	Acute burn RBC's	+	Burn conv. patient serum			→	no agglutination
8.	Conv. Pt's RBC's	+	Conv. Pt. sera	+	C'	→	no hemolysis
9.	Conv. Pt's RBC's	+	Conv. Pt. sera			→	no agglutination
10.	Conv. Pt's RBC's	+	Acute burn Pt. sera	+	GPC'	→	no hemolysis
11.	Conv. Pt's RBC's	+	Acute burn Pt. sera			→	no agglutination

Table 2 (Continued)

12.	Sheep RBC	+	Amboceptor	+	GPC' ⟶	hemolysis
13.	Human "A" RBC's	+	Human O sera (immune 1:200)	+	GPC' ⟶	hemolysis

A dilution of 1:30 guinea pig complement was used for this testing and together with Amboceptor gave 100 per cent hemolysis of human cells. The blood of both patients was O Rh+ so that no isohemagglutinin problem was evident. Kolmer saline was employed with $CaCl_2$ and $MgSO_4$ added. All sera for hemolysin tests were inactivated at 56° C. but uninactivated sera were used for agglutination tests.

From the above results, it is evident that within the experimental limitations imposed no hemolysins or hemagglutinins were detected. However, the possibility that either of these agents were heat-labile was not ruled out.

The same sera from the two patients, uninactivated but Seitz-filtered, was added to HeLa cells grown in Eagle's tissue cell culture medium. Both sera proved toxic for these cells so that it was not feasible to demonstrate an anticytolysin in the postburn serum.

This was the extent of our work with the "burn toxin." We have been

interested in the report by Atherton that among male Wistar rats

receiving a standardized immersion back burn of 32 per cent of the

body surface, a high incidence of positive direct Coombs test in the

period 48-96 hours was associated with death in 24 hours.

B. Specific Hyperimmune Serum for Septicemia Consequent to Burn
    Injury

The failure of antimicrobials to prevent or eliminate Pseudomonemia

in our severely burned patients has caused us to examine the role

of specific antibody for treatment of infection experimentally induced

in animals. It has been demonstrated by active immunization with

various Pseudomonas lysotypes that heterologous strains do not provide

100 per cent protection against another challenge organism. However,

when the immunizing strain was the challenge strain, a single injection

of a living strain given IP to mice was sufficient to give between 80-100

per cent protection. These findings are also referable to autograft

rejection in rats. When the immunizing strain was used to seed auto-

grafts, no rejection of the graft occurred.

Otherwise, the work that this unit has done with hyperimmune sera is

essentially the same and is in agreement with the work of Millican and

Rust. Hyperimmune serum made using live Pseudomonas organisms

or merthiolate-killed organisms is tremendously more effective than the antibody normally occurring in humans, which is to say, as Millican and Rust said, that hyperimmune serum is tremendously more effective than normal gamma globulin.

Our method was to measure by hemagglutination, using PC-9 antigen, the antibody titer to this Pseudomonas polysaccharide in the sera of healthy and severely burned individuals. Then we assessed the protective quality of this antibody by using serum from these individuals to protect mice against challenge by Pseudomonas organisms. The PC-9 antibody was found to be negligibly protective. Serum taken from a human, who, in effect, had immunized himself by a protracted Pseudomonas septicemic episode was, on the other hand, highly protective for mice, as was hyperimmune serum made in rabbits using Pseudomonas. On the basis of this work, we hope ultimately to use hyperimmune serum made in an animal or human for treatment of burned patient septicemia due to Pseudomonas.

Pseudomonas bacteriophagy performed at this unit indicates three important nosocomial lysotypes of high virulence to be responsible for 98 per cent of our Pseudomonemias. It is expected that a polyvalent antigen comprising these three types would be used to immunize

an animal whose gamma globulin might be despeciated for immuno-
transfusion. The possibility of immunizing humans with a merthiolate-
killed Pseudomonas vaccine for recovery of hyperimmune serum is a
possibility that is being investigated currently.

To summarize with respect to the foregoing, it is our opinion that there
is no laboratory evidence from our unit for the existence of a "burn
toxin". A critical analysis of the literature on this subject and an
attempt, in one case, to reproduce another investigator's work
convinces us that evidence is lacking for a "burn toxin" generally.

APPENDIX E

Summary of Investigations
Conducted at University of Texas Medical Branch
Concerning the Presence of a Toxic Antigen
and the Formation of Antibodies in the Burned Organisms

Truman Blocker, Jr., M. D.
August, 1961

Our laboratories have been actively engaged in a major program of study of several aspects of thermal burn trauma since 1950. Since 1955, various attempts have been made to confirm the reports of other authors concerning the proposed concept of an autotoxin-antitoxin phenomenon following extensive burns. We have used numerous animals including dogs, rats, and guinea pigs. Attempts have been made to duplicate the exact protocols of other investigators insofar as could be determined. In addition, several other techniques and methods of study have been employed.

In our most recent work we have used Holtzman albino rats which ranged in weight from 180 to 200 gm. The animals which were burned were subjected to a carefully standardized burn in water at a temperature of 90° C. Ether anesthesia was used exclusively because in the past we have encountered difficulty in maintaining standard conditions using nembutal for anesthesia. We have used a "weight immersion burn technique" which will be described in a forthcoming article.

The burns inflicted have been within the range of 30 to 60 per cent of weight immersion, which may then be related to skin surface area. The treated animals were given convalescent serum, which was administered to one group before burning and to another group after burning.

Under these experimental conditions, we found that the duration of exposure to the heat and the depth of the burn inflicted are more important in causing death than the extent of body surface burned. An exposure for 45 seconds is required to achieve 100 per cent early mortality. At this length of exposure, all controls and treated animals succumb within 24 hours. However, in these experiments it is not known whether the animals died because they were overwhelmed by the traumatic experience, or whether or not the serum had no effect in the treated group. In another series of experiments, it was determined that exposure for 30 seconds yielded inconsistent results.

However, in a series of studies, using an exposure of 15 seconds we found that all animals survived the early postburn period. One group of these animals was treated with serum from rats which had recovered from burns. At the end of one week about 50 per cent

of the animals in both the untreated control and treated groups died.

We are reluctant to draw definite conclusions from these observations

because we believe that the use of ultimate survival as an index for

evaluation is open to serious question due to the many additional

factors which come into play during convalescence after burning.

For example, the wound becomes infected eventually, and the constricting

effect of the dry eschar on the wound limits mobility and, hence, fluid

intake.

We have also injected extracts of burned and normal skin of rats

into other rats by the intraperitoneal, subcutaneous, and intravenous

routes. These extracts were shown to be nontoxic when cleared of

particulate materi al.

We also made limited attempts to duplicate the experiment in which

the Russian scientists reported that they observed anaphylaxis in

guinea pigs injected intraperitoneally with the serum of recently

burned rats. We never observed anaphylaxis, although death did

result within 16 to 24 hours from what appeared to have been a

delayed anaphylactoid reaction.

In addition, we have conducted various in vitro studies in an attempt

to detect an auto-antigen-antibody reaction following burning.

We have demonstrated an increased hemolytic effect of noninactivated serum of burned rats on washed erythrocytes of rabbits in a 1:64 suspension. Hemolysis was noted in dilutions between 1:4 and 1:32. We are unaware of the significance of this. However, serum from normal rats has a tendency to lyse the erythrocytes of normal rabbits. Thermal trauma to the rat seems to result in enhancement of this tendency.

We have attempted to demonstrate agglutination reactions (caused by the serum of burned animals) using the Boyden Tanned Cell Method, but with inconsistent results and considerable technical difficulties.

Precipitation reactions with the Ouchterlony method of diffusion-in-gel and immunoelectrophoresis by the Grabar-Williams method have yielded the most interesting results. In these tests, 13 specimens were used. Eleven of these were serum obtained from recently burned rats; two were extracts of the burned skin of recently burned rats. When these materials were reacted against serum from rats which had received a single burn, only a nonspecific precipitation resulted. When the tests were repeated using material from twice-burned rats, however, precipitation lines were produced in two instances. Although demonstrated by both techniques, the precipitation lines were quite

distinct on Ouchterlony plates but somewhat less well defined in the immunoelectrophoretic preparation. In one of these instances, the material used was serum obtained from a rat within 10 minutes after burning; in the other, the material was an extract of burned skin of a rat taken 24 hours after injury. In no other preparation, including those using material from burned and control animals, were these results observed. The preparations used as controls included hemolyzed and nonhemolyzed blood, normal and heated blood, hemolyzed red blood cells, and plasma.

Evidence that this reaction is a specific one rests upon the fact that the diffuse precipitation observed when "antigen" of a once-burned animal was used, became a distinct line characteristic of a specific response when "antigen" from a twice-burned animal was used. Also, if this is a true antigen, it is apparently cell-fixed or at least is not freely circulating very shortly after burning. Schultz-Dale tests are being done in an effort to obtain further elucidation on this point. So far, one positive reaction in three attempts has been observed.

In summary, we have no evidence of biological activity of a "burn toxin". We have some evidence of an auto-immune phenomenon following extensive thermal trauma, but we are unaware of its significance. That such a phenomenon would come into play is in itself

not considered to be surprising. An extensive thermal burn of the

skin certainly results in denaturation of proteins in the skin and in

the liquid and formed elements of the blood in the traumatized area.

These materials are known to have antigenic properties. Certainly

also conditions prevailing at the site of trauma would make possible

the release into the lymphatic and cardiovascular circulations of

the denatured products of burned tissue. Therefore, it seems

reasonable to expect that a number of antigen-antibody reactions could

take place in the living organism following burning. However, the

significance which these reactions might have is debatable.

APPENDIX F

Summary of Investigations
Conducted at the National Institutes of Health
and the Hospital del Nino, Lima, Peru
Concerning the Presence of a Toxic Antigen
and the Formation of Antibodies in the Burned Organism

Sanford M. Rosenthal, M. D.
August, 1961

Our experience with use of convalescent serum in burns has been limited to animal experiments. However, we feel that there are several points requiring clarification.

Use of convalescent serum is advocated by some investigators as a therapeutic agent against a burn "toxin" which operates as a causative agent in postburn shock. We believe the experiments from which these conclusions were drawn should be repeated under well controlled conditions. Currently it appears to us that it is more likely that the basis of any merit the use of convalescent serum may have is due to the presence of antibodies against organisms involved in postburn infection rather than from an "antitoxin". Our experimental studies at the National Institutes of Health lend support to this possibility, in which we observed that serum obtained from organisms that had recovered from burns was only slightly more effective than normal gamma globulin against delayed deaths in burned mice.

Our clinical studies in the Peru Project indicate that little effect against burn shock may be anticipated from use of convalescent serum in adults. The fact that large doses of saline solutions have produced a zero mortality from shock in over 100 cases of burns of up to 50 per cent body surface militates against a bacterial factor or toxin as a cause of death during the shock phase. It is possible that the antibacterial antibody content of convalescent serum may have some protective value against delayed deaths from burns in adults.

In young children there is good evidence that bacterial invasion (Pseudomonas and Staphylococcus) may begin during the shock period and contribute to early as well as late mortality. While plasma and large doses of gamma globulin have a pronounced effect in children, it is possible that convalescent serum might be more active, and a trial is being conducted in Lima on this score.

APPENDIX G

Comments Regarding Immunotransfusion in the Treatment of Burns
Captain L. F. Miller, MC USN, Officer in Charge
Naval Medical Research Unit #4 (NAMRU # 4)
Great Lakes, Illinois
August, 1961

Pilot studies were attempted to develop a technique for demonstration

of toxin-antitoxin factors postulated by Dr. S. R. Rosenthal of the

University of Illinois. The studies were done in collaboration with

Dr. Rosenthal upon the occasion of the Chicago school fire in

December of 1958, which provided opportunity for utilization of

convalescent serum in treatment of burns. Since 1959, sera from

burned patients provided by Dr. Rosenthal and Dr. Kenneth Sell of

the Naval Medical School, National Naval Medical Center, have been

tested at NAMRU #4 with the tissue culture technique developed by

Dr. Max Rosenbaum.

Sera from acutely and extensively burned children who were hospital-

ized at St. Anne's Hospital as a result of the school fire were observed

to be inhibitory to the outgrowth of HeLa cells from the seed, whereas

sera from less extensively burned children and sera from nonburned

children obtained at Great Lakes as controls were not. Based on a

single titration, a pool of convalescent sera obtained from three

previously burned donors was three- to fourfold more effective in

neutralization of growth inhibition than a pool of sera from normal donors. Insufficient inhibitory burn sera were available for titration of neutralization by individual normal serum specimens to determine the range of expected neutralization titers of sera from normal donors. Consequently the three- to fourfold difference observed for the convalescent pool does not provide a basis for determining whether sera from convalescent donors had higher neutralization titers than would be expected from sera obtained from normal donors. Gamma globulin fractions obtained by electroconvection from convalescent and normal sera showed the same relationship in neutralization titers as the sera from which the fractions were obtained.

Children from whom the growth inhibitory sera were obtained, when bled following transfusions of convalescent blood or plasma, provided serum that was no longer inhibitory, suggesting the possibility that some sort of neutralization was occurring in the patient which had not occurred following multiple transfusions with normal blood or plasma. The validity of this finding is uncertain due to the fact that we have since, in confirmation of Dr. Sell's observations, observed that serum from normal blood becomes inhibitory when stored in contact with the clot at room temperature for more than 24 hours.

Although blood from the burned children studied in 1958 was probably placed immediately in the refrigerator and kept there until shipment to NAMRU #4, there can be assurance on that point since the hospital was operating under emergency conditions. We are not satisfied that storage variables were sufficiently controlled to rule out fortuitous effects from methods of specimen handling before testing. Furthermore, we have no information to indicate the nature of the factors responsible for growth inhibition. For example, the pH was not accurately determined and variations of this factor can easily affect outgrowth. Evidence of hemolysis was usually present but degradation products of the blood were not looked for. However, the serum from hemolyzed normal blood did not result in inhibition. E. Coli endotoxin and histamine in higher than maximum concentrations expected in burn patients did not cause inhibition.

Relative to clinical conditions and inhibition of cell growth by serum, there was a gross correlation between inhibition and the degree of burns. It was demonstrated that sera from less extensively burned children were not inhibitory even though the specimens were presumably processed by the hospital staff in a manner similar to that used for the sera from the more extensively burned children. Under the circumstances, however, it was not possible to obtain adequate

clinical data. These data were largely subjective impressions which were not obtained on a double- or single-blind basis. Other variables could not be determined or controlled to determine the significance of the loss of inhibition of sera collected after transfusion of convalescent blood or plasma. Adequate bacteriological data necessary to even attempt correlations with observed effects in tissue culture and presence of sepsis and wound infections were not obtained.

Subsequent to testing the sera from children hospitalized at St. Anne's a number of sera were tested in collaboration with Dr. Sell of the Naval Medical School. Our results have confirmed his. Clear-cut evidence for inhibitory sera from burned patients has not been obtained, although there were relatively few specimens collected comparable to those obtained from St. Anne's in respect to age, extent of burn, time of collection postburn, and storage conditions. In addition, the original HeLa strain used became contaminated necessitating use of a new HeLa cell line which may not be as sensitive.

Conventional serological techniques, such as complement fixation and hemagglutination tests, were inconclusive and positive results were never more than border-line. The majority of specimens gave negative results. Furthermore, the degree of growth inhibition and

neutralization of this effect observed in the tissue culture technique
were of low magnitude.

In Summary: The current status of results obtained by NAMRU #4 in
studies on the serum of children burned in the school fire of 1958
is one of uncertainty. Data obtained with the tissue culture technique
are consistent with but are in no way confirmatory of a toxin-antitoxin
concept. The manner in which inhibition is produced and the effect
of variables introduced in the collection, storage, and processing
of specimens are unknown. This situation does not allow confidence
in the data obtained. Inadequate amounts of inhibitory sera were avail-
able to determine neutralization titers of normal and convalescent
sera and thus demonstrate whether sera of convalescents have
unique neutralizing substances. Objective clinical data could not be
obtained and subjective clinical data were not collected in a single-
or double-blind manner. Controlled data were impossible to obtain
under the circumstances. Many other treatment variables were
present and their significance cannot be determined.

APPENDIX H

Report of Personal Visits
to Soviet Burn Centers and Comparison
of Certain Data on Burn Patients

Virginia Blocker, M. D.
August, 1961

In June, 1960, I attended an International Symposium on Plastic Surgery

in Marianske Lazne, Czechoslovakia, at which Dr. Ludmila Pushkar,

a surgeon at the Central Institute of Hematology and Blood Transfusion

in Moscow, made a report concerning clinical use of convalescent

burn serum. This work was done in conjunction with Dr. Gruzhdova

under the direction of Professor N. A. Feodorov. Dr. Pushkar's

paper appeared to be more detailed than any previously published on

this subject in the Russian literature. Some of the slides were in

English, and the work on treatment of dogs with convalescent serum

following experimental burns was particularly convincing as measured

by mortality figures in relation to the control series. In clinical

patients, results were much more difficult to evaluate because there

were many variables in treatment, and this Pushkar admitted. However,

she reported a very large control series and stated that every effort

was being made to analyze results to avoid being over-enthusiastic.

She stated that convalescent blood and serum are being collected in

250 to 450 ml. amounts from burn patients up to a period of six

months following complete coverage with skin or spontaneous healing of the burned area. These donors are given replacement transfusions if indicated. Intravenous injection of serum is given to the burned patients for the first two days, followed by blood wherever possible, with amounts varying from 100 to 400 ml. per day and continued for approximately one week. In private conversation Dr. Pushkar was most enthusiastic about the response of burned patients to immuno-transfusion during the shock period and again during the second week.

Dobrkovsky has sent us the advance proof of Pushkar's paper which may have appeared by now in the Czech Plastic Surgery journal. Unfortunately, it does not include the work of Feodorov, and Pushkar's portion has been considerably de-emphasized with tables omitted, particularly those of the control clinical series. Nevertheless, there are some interesting statistics included, which has made it possible to make a comparison with a series of statistics of our own, covering 1,000 burn patients. From this analysis it seems that our burn problems and theirs are different in many respects. Table 1 shows a comparison of our cases.

From the cases reported by Pushkar and by other Soviet surgeons, it seems that their burns are not as extensive as ours nor of as great

severity with respect to depth. However, at the 50 per cent level,
more deaths occur in the Soviet series. Pushkar has state dthat one-
third of the burn deaths occur in the shock period, but that with use
of convalescent serum, deaths have dropped to approximately 25
per cent. In this country about 5 per cent or fewer of fatal cases
succumb in the first 48 hours, irrespective of supportive therapy.

I also visited Dobrkovsky's unit in Prague and saw a number of patients
who had been treated with convalescent immune serum. At that time
there had been a total of 30 cases, and in the laboratory we saw data
of Dr. Pavkova, head of the Transplantation Laboratory, who had
been plotting curves of rise and fall of antibody levels. The Czech
workers stressed the fact that positive tests were obtained with unin-
volved skin in burn patients but not with skin of unburned controls.
Dobrkovsky's patients appeared to us to be receiving good care, and
we were most impressed with their state of nutrition and high morale.
I discussed the use of convalescent serum with several Czech plastic
surgeons, including Karfik of Brno. All emphasized that it did not
affect over-all mortality and stated that they had too few cases as
yet to evaluate it properly.

Table 1

Statistics on Pushkar's Series
Treated with Convalescent Serum[a]

Extent of Burn	Patients	Deaths
10 - 40%	86 (69%)	2 (both over age 65) 2. 4% during toxemic phase
41 - 50%	26 (21%)	--------
Over 50%	11 (10%)	1 death during "toxemic" phase, 9 deaths from "sepsis"[b] 90. 9%
Total	123 (100%)	12. (9% total mortality)[c]

a. Taken from a total of 950 patients (Information on depth of burn not included)

b. Not included in series of 114 in article

c. 25% of deaths occurred during the early period in comparison with about 35% in the control series during this time.

Blocker's Series

Extent of Burn	Patients	Deaths	
10 - 40%	575 (55. 7%)	31	5. 3%
41 - 50%	62 (6. 2%)	12	19. 3%
Over 50%	111 (10, 1%)	81	73. 0%
Total	748[d]	124	(16. 5% of this group)

d. 252 patients with 1 - 10% not included (28% of series); mortality in this category was 1. 6%

Table 1 (Continued)

Mortality Rates Compared

	10%	20%	30%	50%	70%	Over 70%
Dzhanelidze	.2	3.9	27.3	70.0	100.0	100.0
Postnikov	.3	2.2	16.3	51.9	63.9	94.1
Pushkar	1.3	1.2	--	38.4	40.0	85.0

Blocker	1.6	1.5	4.0	19.3	77.2	94.7
				40%	60%	
				17.3	54.9	

APPENDIX I

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TREATMENT OF SEVERE BURNS WITH HOMOLOGOUS SERUM

OF RATS CONVALESCING FROM BURNS OR

FROM OPEN WOUNDS

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In spite of progress in the treatment of shock, improved dressing and grafting procedures, chemotherapeutic agents and antibiotics, the mortality from burns involving more than 40 percent of skin area remains high. Especially; the use of antibiotics and chemotherapeutic agents since 1942 has not resulted in significant lowering of burn mortality (Altemeier and MacMillan, 1960). The demonstrated in-ability of available antibiotics to effectively combat infections with Gram-negative bacteria, especially Pseudomonas aeruginosa, may be one of the main obstacles to successful treatment of severe burns. On the other hand, it is well recognized that the success of antibio-tics in combating infection is dependent not only upon bacteriostatic and/or bactericidal properties of the drugs, but also upon humoral

and cellular defense mechanisms of the body.

It has been postulated, but not conclusively proved, that potentially toxic factors unrelated to infection arise in burned tissue. These "burn toxins" are supposed to be antigenic so that the "burn toxemia" may be counteracted by the development of specific antibodies. A corollary of this hypothesis is that the serum of surviving burned patients or animals whose burns have healed, will have developed antitoxin titers sufficiently high to permit passive immunization. There is some evidence that burn toxins may not be species-specific; if so, not only homologous but also heterologous passive immunization might be possible. Results reported by some investigators using burn convalescent serum, seemed encouraging. However, few experiments have been well-controlled, and the possibility has not been investigated that the mechanism by which burn convalescent serum protects severely burned animals may be due to antibacterial rather than antitoxic activity of the serum, or possibly. a combination of these activities.

The experiments reported here were undertaken (1) to evaluate the effectiveness of sterile burn convalescent serum in reducing mortality from severe burns in the rat and (2) to compare this serum

with sterile serum from rats convalescing fromsurgical excision of skin areas equal to that thermally injured in rats donating the burn convalescent serum.

The rationale of using serum from rats with excised wounds was provided by observations made during experiment I reported below. When rats with excised skin were kept in the same racks as rats burned to yield burn convalescent senum, the bacterial flora of the wounds was identical in the two groups of animals, beginning three weeks after excision and burning. Since both groups of rats had to cope with the same wound flora, they presumably would have produced identical antibacterial antibodies to combat the infection. In pilot studies, Muschel (Division of Serology, Walter Reed Army Institute of Research) found that sterile pooled sera from the rats used to donate burn convalescent serum and serum after skin excision had higher antibacterial titers against certain pathogenic test bacteria than the sterile pools of normal rat serum. No differences were found in properdin levels between normal sera and convalescent sera.

Throughout these experiments a blind or double-blind randomized design was used to avoid bias as far as possible.

REVIEW OF THE LITERATURE

The earliest reports of therapeutic trials of convalescent burn serum were those of Schütz in 1934 and 1935 and Segal and Uzdin in 1940. Homologous convalescent serum was used from repeatedly burned rabbits and from burn patients (Schütz) and from convalescent severely burned dogs or patients (Segal and Uzdin). Good results were claimed but the reported methodology was not detailed enough for adequate evaluation. More recently Feodorov and Skurkovich in 1955 reported therapeutic results with (1·) convalescent serum obtained from dogs burned once or repeatedly; 9 - 15% surface area, (2) with iso-immune serum prepared by giving repeated intravenous injections of blood from severely burned dogs bled-in the early phase after burn, and (3) with hetero-immune serum obtained by immunization of goats and rabbits with antigen prepared from dog skin burned in vivo. Test dogs were flame-burned over 40% body surface for 1 1/2 or 3 minutes without anesthesia. Immune sera-(1-1 1/2 body weight) were injected daily for 5-7 days after burn, resulting in immediate and long-term reduction in mortality. Dogs burned for 3 minutes had a pooled 24-hour mortality of 4% (77 animals) when treated with immune sera, and of 81% (31 animals) when treated with serum or blood from unburned donors, or dextran or other colloids. In groups burned for 1 1/2

minutes, 9 dogs treated with homologous serum from unburned donors
and 5 animals treated with serum from unburned goats died in 7-12
days; by contrast, 7 dogs treated with convalescent dog serum were
all alive at 36 days, 5 out of 6 dogs treated with iso-immune serum
survived 36 days, and 5 dogs treated with hetero-immune serum all
survived 12 days but were dead at 36 days. Grosdov, Pushkar and
Rovnov (quoted by Feodorov and Skurkovich) treated burned patients
with human convalescent burn serum claiming that "immunotherapy
permits to cure extremely heavy, apparently hopeless cases of
second to fourth degree burns of 50-52% surface area." Skurkovich
and Zaretskiy reported in 1959 that in seven severely burned dogs
convalescent serum therapy prevented anuria and improved renal
circulation; furthermore, repeated thermal injury damaged renal
tubular function less than the initial burn.

Millican and Rust (1960) used homologous convalescent serum in
mice scalded over two-thirds surface area and treated with 0. 9%
NaCl solution (15% body weight) to combat shock. Serum was ob-
tained at 3 to 8 weeks from mice convalescing from scalds, and
compared with human gamma globulin, chloramphenicol and saline
alone. Chloramphenicol was most effective in lowering mortality,
human gamma globulin and homologous convalescent serum less

effective. Gamma globulin was beneficial only during the first week, convalescent serum through 3 weeks. The initial response was nearly identical with human gamma globulin and with mouse convalescent serum (1-2% body weight). Combined therapy with convalescent serum and chloramphenicol had an additive effect.

6. R. Rosenthal whose first publications on toxic factors in burns appeared in 1937, reported in 1960 that blood or plasma of burned-and-healed human subjects transfused into acutely burned children markedly improved their clinical condition, decreasing pain, edema, fever, and urinary albumin.

EXPERIMENT I

Preliminary studies done at the Medical College of Virginia (Richmond, Va.) were reported by one of us (O. J. M.) in 1958 to the Committees on Shock and Trauma, National Academy of Sciences - National Research Council. Male Sprague-Dawley rats, 300-325 gm., were used. Sterile convalescent burn serum was obtained 2 months following a 25% full-thickness burn using a standardized hot-plate technique with burning temperature of 480° F (250° C.). (Details of the burning procedure will be reported elsewhere). Serum was also harvested from rats 2 months after surgical excision of 25% surface area of

dorsal skin ("wound serum"). Sterile normal serum was obtained from unburned rats. As in all subsequent experiments, sterility of sera was insured by cultures (aerobic and anaerobic) before and after pooling of sera. The test-burn was a 50% surface area, full-thickness burn under short-lasting ether anesthesia, 24 hours after close clipping under Nembutal anesthesia. All rats were housed individually in wire-mesh cages, in an air-conditioned room. The 3 types of sterile sera were mixed individually immediately prior to injection with equal amounts of sterile 6% dextran in 0.9% sodium chloride solution. Injections were given intraperitoneally (1.5% body weight of serum and 1.5% body weight of dextran/saline) immediately following burns, still under anesthesia. No food or water was allowed for 24 hours after burning; then food and tap water were given ad libitum.

Results: As seen in Table I, convalescent serum (one dose only, 1.5% of body weight injected intraperitoneally immediately after burning) lowered the mortality significantly on days 3 through 12 following burn, as compared with normal serum from unburned rats and with dextran/saline treatment alone. "Wound serum" in the same dosage reduced mortality on days 3 through 5, but with no statistical significance compared with normal serum. On days 8 through 12,

cumulative mortalities after treatment with normal serum, "wound serum" and dextran/saline were almost identical, all animals dying by day 12. Rats treated with one injection of convalescent burn serum deteriorated rapidly on day 13 and were all dead by day 20.

MODIFICATIONS IN METHODOLOGY

At the Walter Reed Army Institute of Research (WRAIR), the burning technique described above proved insufficiently severe for the WRAIR strain of pathogen free Wistar rats. In Richmond (M. C. V.), the mortality following a 50% surface area, full-thickness burn treated with dextran/saline only (3% body weight) was 95% of 100 rats at 10 days and 100% at 12 days, but the mortality for identical burns and treatment at WRAIR was 6% and 6% of 100 rats respectively. Strain difference and/or different bacterial flora in the 2 locations may serve as explanations. Burn eschars began to slough much earlier in the Richmond rats (5th day) leaving wet, foul-smelling wounds heavily infected with Proteus and other Gram-negative bacilli, while eschars in the WRAIR rats did not begin to slough until the 14th-20th days and the resulting open wounds had dry, non-smelling granulations with a flora of E. coli, Streptococci and Staphylococci.

Only occasionally was Proteus cultured from wound smears at WRAIR.
Such difference in flora can be expected since the WRAIR rats are
reared pathogen-free and all rats used for test-burning were kept in
a clean, air-conditioned room (previously a chemical laboratory);
no other animals are housed there. The animal room used in Richmond
contained several animal species and was subjected to much traffic.
To attain greater mortality from thermal injury in the WRAIR rats, the
burning temperature was increased to 500° F (260° C.) and time of
contact of the dorsal and lateral skin with the hot surface was increased
from 7 to 8 seconds. Furthermore, after clipping a chemical depilator
(Nair) was used; after 2-3 minutes the remaining hair was shaved
off, leaving a completely hair-free, smooth skin surface. Burns
which resulted were full-thickness in the true sense, involving coagu-
lation of the cutaneous muscle layer down to the subcutaneous fat
but not injuring the underlying skeletal muscles. Treatment with
sterile clinical dextran/saline solution (30 ml/kg body weight intra-
peritoneally) gave a 1-day mortality of 23%, a 2-day mortality of 69%,
and a 3-day mortality of 77%, while the mortality without colloid/
saline treatment was 87%, 93%, and 93% respectively. More effec-
tive treatment during burn-shock phase was clearly necessary for
long-term evaluation of the therapeutic effects of convalescent serum.

After many trials, subcutaneous injection of sterile 0.9% NaCl solu-tion, 14% body weight (7% body weight administered immediately after burning and 7% given 3 hours later) was found satisfactory, resulting in 1, 2 and 3 day mortalities of 3, 15, and 25%, respectively.

These modifications have been used in all subsequent experiments.

It is emphasized that, throughout, rat batches treated with the different types of sera were equalized with regard to body weight (mean pre-burn weight and spread around the mean); furthermore, the same person has done all test-burning procedures and the animals were selected for treatment with the different types of sera on a randomized basis or by coin-tossing. To rule out bias as far as possible the person performing the burning did not know at the time of burning what type of post-burn treatment was selected for the individual animals.

EXPERIMENT II

Two series of test-burns were done 3 weeks apart. Series IIa con-sisted of 15 animals treated with normal serum and 14 animals treated with convalescent serum. Injections of serum were given intraperitoneally: 1% body weight immediately after burn and 0.3%

on days 4 and 7 after burn; a total of 1.6% body weight in the first week. Series IIb involved·16 rats treated with normal and 15 with convalescent serum. The·doses were 1% body weight on day 2 and 0.6% body weight on day 6 following burn, the total dose administered during the first week being the same as in Series IIa. The batches of sera were identical in both experiments. Sterile convalescent serum was obtained 5 months after one burn, full-thickness, of 25% body surface-area, from rats of the pathogen-free WRAIR strain used for test-burns. The burns were completely healed in all donor rats. All animals were kept in individual cages as described earlier. No significant differences in mortality were observed between Series IIa and IIb, so the 2 runs have been pooled and the results are summarized in Table II.

Animals treated with convalescent serum had lower mortality than those treated with normal serum. The differences, however, only border on significance, at 2d, 3d, and 5th weeks after burn (p less than 0.05).

EXPERIMENT III

This experiment was designed to study the long-term effects of treatment with different types of convalescent burn sera. To investi-

gate the possibility that injections of sterile complete Freund's adjuvant (obtained through the courtesy of Dr. E. Becker, Walter Reed Army Institute of Research) might increase the rate of antibody formation in repeatedly burned rats, intramuscular injections of adjuvant were done with the first and fourth burn in sites adjacent to the burned area. One group of unburned rats was injected intramuscularly with Freund's adjuvant 15 days apart and bled one month following the first injection. Serum from these animals was used to ascertain whether injections with adjuvant produced a therapeutic serum more or less effective than the normal rat serum used as control for the convalescent burn sera.

Three series of test-burns were performed under identical randomized conditions, one week apart. In each series six sterile pooled sera were used on groups of 13-14 animals each. The six types were:

A. Convalescent serum, collected 3 months after 1 burn (25% body surface); donor animals kept in clean room at Walter Reed. The burn wounds were healed in the majority of rats at time of bleeding and sacrifice.

B. Normal serum from unburned rats, housed at Walter Reed.

C. Serum from unburned rats, given 2 intramuscular injec-

tions of 0. 3 ml. of sterile complete Freund's adjuvant 15

days apart and bled 1 month after the first injection;

donors housed at Georgetown University, Washington,

D. C.

D. Burn serum collected 1 month after the first and 10
days after the last of 5 burns (5% body surface each)
inflicted 5 days apart; donors housed at Walter Reed.
The first two or three burn wounds were healed, while
the remaining wounds were still open at time of bleeding.

E. Same as Group D; donor animals burned and housed at
Georgetown University.

F. Same as Group D and E; donor animals given intramuscu-
lar injections of Freund's adjuvant as in Group C, with
the first and the fourth burn (days 1 and 15); housed
at Georgetown University.

The first dose of serum (0. 5% body weight) was given intraperitoneally
immediately after burn. The second dose (0. 4% body weight) was
injected 24 hours, and the third dose (0. 3% body weight) 48 hours
following burn, both subcutaneously in unburned areas. Later injec-
tions (0. 2% body weight) were administered intramuscularly on
days 3, 6, 10, 13, 17, 20, 24, 27, 31, 38, 45, 52 and 59 following

burn. The total dose during the first week amounted to 1.4% of body weight (or for comparison: 980 ml/70 kg. man), and the total dose in animals which survived on day 59 was 3.6% body weight (or 2520 ml/70 kg. man).

Results (see Table III): As in Experiment II, the survival trend was consistently in favor of the animals treated with convalescent serum obtained, in this case, 3 months after 1 25% burn. The differences between convalescent and normal homologous serum (groups A and B) were significant at 2, 4, and 5 weeks following burn (p < 0.05). On the other hand, the convalescent serum could not prevent a steady rise in mortality after the 5th week; when serum treatment was discontinued at 2 months, mortality in Groups A and B was similar (55% and 60% respectively), .

A comparison can also be made between the 3-month convalescent serum (Experiment III, Group A) and the pooled Groups D, E, F (animals treated with sera from rats burned repeatedly, collected 1 month after the first small burn, 10 days after the last thermal injury). The cumulative mortality figures for Groups D, E and F (Table III) are almost identical at all times following burn, justifying such pooling. The differences in survival rates are strongly in favor of

the 3-month convalescent serum from the second day through the
second week: on day 2 the p value is less than 0.01, on day 3 less
than 0.001, on days 4 through 7 and at 2 weeks less than 0.01,
and at 4 and 5 weeks less than 0.05.

A comparison between the one-month serum from repeatedly
burned animals and normal serum reveals that survival rates with
normal serum treatment are significantly higher on days 2, 3 and 4,
at the 5, 1 and 5% levels, respectively. These findings indicate
that 3-month convalescent-burn serum is certainly more effective
in preventing death during the first two weeks than is 1-month con-
valescent serum from repeatedly burned rats, and, further, that
on the second through fourth days following burn, the latter serum
may actually be harmful as compared with normal serum. No
significant differences in mortalities were observed at any time
after burn between rats treated with normal serum from unburned
donors versus serum from unburned donors injected twice with
complete Freund's adjuvant. (Group B versus Group C). Likewise,
almost identical results were obtained with sera from repeatedly
burned donors (Groups D and E) regardless of location of housing
(Georgetown University versus Walter Reed Army Institute of
Research). . Injections of Freund's adjuvant (Group F) did not

alter the properties of the serum in a way that influenced the survival of burned and treated animals.

EXPERIMENT IV

This experiment was designed as a randomized, double-blind study in order to rule out bias as far as possible. Three types of sterile homologous sera were evaluated in the burn-preparation described previously.

1. 3-month burn convalescent serum tested in a total of 115 burned rats.

2. 3-month wound convalescent serum (serum from rats collected 3 months after about 25 percent surface area of dorsal and flank skin had been excised surgically down to the fascia). Tested in a total of 114 burned rats.

3. Normal serum, injected in a total of 115 burned rats.

The three types of sera were designated A, B, and C and transferred aseptically from 1 liter mixing bottles, code-labeled by a person not connected in any way with the experiment. The code-message was stored in sealed envelopes, inaccessible to experimental personnel until the experiment was finished. The experimental animals were randomized as in previous experiments. Doses of serum were

administered as in Experiment III.

Results: The mortality rate at 48 hours (attributable to shock) was higher in the first four series (burned 55-65% surface area) than in previous experiments. The higher mortality in the shock phase may be attributed to seasonal variations in resistance to stress. However, a more likely explanation for the higher mortality is the lower relative humidity in series IV leading to a higher insensible water loss through evaporation. While the room temperature in all experiments was kept within the range 23.0-25.0° C., the relative humidity in series II and III, performed in spring and summer, was 70-80%, while in series IV, done during the winter, the relative humidity was only 30-40% on the average.

It is well known that the desiccated skin following a full-thickness thermal injury is much more permeable to water than living skin. (Lieberman and Lansche, 1956). An attempt to lower the high 48-hour mortality by reducing the skin area burned from 55-65% to 40-50% was successful. The 48-hour mortality was halved, but there was no protective effect of convalescent burn serum in these rats with smaller burns.

Analysis of the differences in mortality when all rats in each of

the three treatment groups were pooled (burns from 40-65% surface area), showed no statistically significant results. Since the major difference between experiment IV and the previous experiments was the greatly increased mortality of the first 48 hours, and furthermore convalescent serum had no effect on mortality in the first two days, it was deemed justifiable to analyze subsequent mortality on the basis of the total number of rats surviving after 48 hours. It was felt that this would be the only fair way to evaluate the protective effect of the sera, if any, and that this method would also provide a justification for pooling the results of these experiments with previous data.

Tables IV and V show the results and the statistical significance of the data.

Pooling all rats from experiments II, III and IV, indiscriminately, without excluding the smaller burns (40-50%) or those animals which died in the first 48 hours, the lowering of mortality due to injection of burn convalescent, as compared with normal, serum borders on statistical significance in the 4 to 14 days after burn (p = 0. 05 to 0. 03), Table IV, A. Taking only 48-hour survivors from experiment IV, burned 55-65%, borderline significance

is achieved at days 4 and 7 with even the small number involved in
such analysis, Table V, B; on the other hand, a similar number of
rats burned 40-50% only, showed no difference between treatment
with normal and burn convalescent serum, Table V, C. When all
rats burned 55-65% skin area, from Experiments II, III, and IV,
are pooled on the 48-hour survivor basis, the difference between the
mortality of rats treated with burn convalescent serum and those
treated with normal serum becomes significant ($p < 0.01$), Table IV,
C. While the difference in cumulative mortalities is not striking,
it is remarkably consistent, occurring in any consecutive series of
40 rats and up.

Comparison of "wound excision convalescent serum" with normal
serum revealed a significant increase in mortality at 48 hours in
rats treated with wound convalescent serum ($p = 0.02$), Table V, A.
It is of interest that a similar increase in mortality in the 48-hour
period after burns occurred in Experiment III when sera were used
which were obtained one month after the initial burn from rats
burned repeatedly (every 5 days, 5 times), (Table III, Group D,
E and F).

Repeated injections of sterile homologous normal serum were not

injurious in the severely burned animals as shown during series IV

in which one group of burned rats was injected with sterile 0. 9%

NaCl solution in the same volume and at the same time as other

rats received serum treatment. No significant differences in cumu-

lative mortalities occurred, except at day 4 post burn where the

mortality in the rats injected with normal serum was less than

for the saline controls. The difference was, however, significant

only at the 5% level.

DISCUSSION

Previous studies in experimental animals and in patients have

indicated that treatment with parenteral injections of serum or

blood obtained from burned and convalescing or healed donors has

a beneficial effect on vital-signs and survival. (Schutz 1934, Segal

and Uzdin 1940, Feodorov and Skurkovich 1955, Skurkovich and

Zaretskiy 1959, Millican and Rust 1960, and Rosenthal, Hartney

and Spurrier 1960).

The protective effect of convalescent burn serum is postulated to

arise from its content of antibodies towards "burn toxins", formed

in or released from thermally injured tissues and acting as antigens

inducing formation of specific antibodies in the burned organism,

The nature of "burn toxins" has not been conclusively clarified.
Experiments by Wilson (1935) and Wilson and his associates (1936-37),
repeated and confirmed by Cullumbine, McDonald and Simpson (1947)
indicated that burn edema fluid collected 48 hours after burn, but
not at an earlier time, is toxic and may be lethal when injected
into unburned animals in sufficient doses. Extensive studies by
Simonart and his co-workers, summarized by Simonart (1958),
have provided evidence that the toxicity of burn edema fluid, found
in the euglobulin fraction, may be due to the high proteolytic
activity of the edema fluid. The ingenious cross-circulation and
amputation experiments of Cristophe (1939) also constitute a link
in the chain of evidence which supports the concept that toxic sub-
stances do arise in burned tissues. Whatever the nature of the
potentially toxic antigens, studies by Schütz (1935), Feodorov
and his associates (1960) and by Chaet (1960) indicate that the
toxic antigens may not be species-specific and that the possibility
thus exists of passive immunization of burn victims with hetero-
logous sera.

Dobrkovsky, Dolezalova and Pavkova (1960) found specific anti-
bodies against burned skin antigen in burned patients using a
collodion-agglutination method. Rosenthal and his associates (1960)

have presented evidence that antitoxic-like substances active against "burn toxin" are present in the blood of healed burned persons. They used three different methods to demonstrate the presence of "anti-toxin"; neutralization of the cytotoxic effect of "burn toxin" on HeLa cell cultures, hemolysins and precipitins. Feodorov (1960) reported the presence of an antigen in the skin and blood of burned animals (dogs, rats, rabbits, horses) and of human burn victims, which could not be found in healthy subjects. Serum of burned dogs fixed complement in the presence of burned skin antigen, from the seventh day after burning, with maximal titers (1/640) on the 20th day. Complement fixing activity remained for an extended period of time in surviving dogs. The present series of experiments demonstrate in rats that homologous convalescent serum from animals surviving a 25% full-thickness burn has some protective effect in severely burned rats. Protective sera have been obtained at 2, 3, and 5 months following burns. In rigidly controlled, blind and randomized type studies, the results have been reproducible from series to series although it should be stressed that, in our ex-perience, a significant protective effect has only been seen when the full-thickness test burns exceed 50% surface area. Convalescent serum, administered intraperitoneally immediately following burning

in a dose of 0. 5% of body weight, followed by 0. 4% of body weight
24 hours later, had no significant effect on mortality during the first
24 or 48 hours. All rats received 14% of body weight as sterile
isotonic saline subcutaneously during the first 3 hours after burn.
This lack of effect of convalescent serum on the early shock-phase
mortality is in contrast to the report by Feodorov and Skurkovich
(1955) in which homologous convalescent blood or serum markedly
reduced mortality of 40 percent flame-burned dogs already during
the first 24 hours after burn. Our experiments also demonstrated
that a surgically created skin wound of 25% surface area does not
yield a protective serum against burn "toxicity" and that such serum
may actually be harmful in the early post-burn period when compared
with normal serum and especially with burn convalescent serum.

The present experiments in rats provide only indirect evidence in
support of the toxin-theory of burn autointoxication. Pilot studies
of antibacterial antibody activity indicate that both convalescent burn
serum and serum obtained after skin excision have increased titer
of certain antibacterial antibodies while properdin levels are not
different from those found in unburned control animals. The crucial
experiments to separate antitoxic antibody activity from antibacterial
antibody activity as a factor in the demonstrated protective effect in

severe burns remain to be done. Conclusive evidence in support
of the "burn toxin" theory could be obtained by experiments with
germ-free mammals if it were shown that convalescent serum from
unburned germfree animals had a protective effect in severely
burned conventional animals in the absence of specific antibacterial
antibodies in such serum towards the pathogenic wound flora in con-
ventional animals. Vice versa, support for the toxin theory could
also be furnished if sterile convalescent burn serum from conventional
animals were effective in severely burned germfree animals which
would not need antibacterial antibodies in the total absence of
bacterial flora.

Another interesting finding in the present experiments was that al-
though it has been reported (Schutz 1934) that repeated small burns
may protect an animal against a later severe burn which would be
lethal in control animals, we could not demonstrate any protective
effect of one-month burn convalescent serum produced by repeated
small burns with collection of serum 10 days following the last
burn. On the contrary, like the serum obtained from rats convales-
cing from surgical excision of skin, this one-month convalescent
burn serum was probably harmful in the early shock-phase after
burn. A possible explanation for this lack of protective effect already

implied by Rosenthal et al. (1960) in another context, is that al-
though antibodies may be formed after each small burn, each thermal
insult gives rise to newly formed antigen which after being absorbed
into the circulation is neutralized by antibody. If the time lapse
after the last antigen release to collection of serum is too short,
as it may have been in our experiment, an antibody-saturation
effect may explain the absence of free anti-toxic antibodies in the
one-month serum and lack-of protection following injection into
burned test-animals. In the same vein, one may also hypothesize
that the deleterious effect of the one-month convalescent serum
might actually have been due to its content of some unneutralized
"toxin". Further studies should clarify these points.

Other observations emerging from our studies were that injections
of complete Freund's adjuvant with the first and the fourth of five
repeated small burns did not alter the therapeutic results with the
one-month convalescent serum; nor did injections of adjuvant into
unburned rats result in a serum-different in therapeutic properties
from normal rat serum. Furthermore, repeated injections into
burned rats of normal homologous serum were not deleterious com-
pared with sterile isotonic saline administered in the same volume
and time schedule. Finally, treatment over a period of 8 1/2 weeks

with convalescent burn serum obtained 3 months after one 25% burn, was ineffective after the fifth postburn week when compared with normal serum treatment. ·Incomplete and limited evidence exists that in our rats, septicemia (blood cultures taken from the abdominal aorta under strict asepsis) was present in about half of the moribund rats from the second through the ninth postburn week.

Comprehensive bacteriologic – immunologic studies are clearly needed to justify many as yet unproved assumptions in order to provide a sound basis for treatment with convalescent burn serum in experimental animals and in human patients.

SUMMARY AND CONCLUSIONS

1. A randomized, blind or double-blind experimental design has been used to study the therapeutic effect of sterile, homologous convalescent burn serum in severely burned adult male rats of ordinary, or in the large majority of studies, of a pathogenfree stock. The standardized test-burn used to evaluate therapeutic effects of sera was a high-temperature contact burn (250° and 260° C.) of depilated skin; the time of contact with thermocontrolled hot-plate was 8 seconds on back and flanks, 3 seconds on abdomen.

This thermal insult done under ether anesthesia produces a full-thickness burn of all skin structures without injury to underlying skeletal muscle.

2.   Convalescent burn sera were produced in the same stocks of rats by inflicting a 25% surface area full-thickness burn and bleeding the animals 2, 3 or 5 months after thermal injury.   In one experiment, repeated small burns, each about 5% surface area, were produced 5 days apart, 5 times, resulting in a total of 25% surface area burned by day 20.   Blood was collected one month after the first burn.   In one series of the same experiment, sterile complete Freund's adjuvant was injected with the first and the fourth of the repeated burns, in the vicinity of the burned area.

3.   In two experiments, a 25% surface area dorsal skin excision down to the fascia was done under Nembutal anesthesia in rats which were bled 3 months after surgery.

4.   Control serum from unburned rats was obtained by bleeding normal rats.   Blood for production of all types of sera was drawn under aseptic conditions from the abdominal aorta under light Nembutal anesthesia, cultured and recultured after pooling of corresponding batches to assure sterility.   Sera were stored at -20° C. until used for injection.

5.   Burn wounds and the wounds of surgically excised rats donating sera in Experiment I were heavily infected with an identical mixed flora of which Proteus and other Gram-negative rods were prominent. In this experiment a significant protective effect of one dose of 1.5% of body weight of burn convalescent serum injected intraperitoneally immediately after burn was seen in the 50% surface area test-burned rats, when compared with burned rats injected with either serum from skin-excised rats, normal (unburned) rats, or with dextran/saline only. The burn convalescent serum and the serum from skin-excised rats were obtained 2 months after burning and excision, respectively.

6.   In Experiment II, rats test-burned 60-65% surface area at 260° C. received burn convalescent serum, collected 5 months after one 25% burn. Controls received normal serum. Sera were administered to one-half of each group in a dose of 1% of body weight immediately after burn, followed by injections of 0.3% of body weight on the 4th and 7th days after burn; the other half of each group received one injection of 1% of body weight of serum on day 2 and 0.6% of body weight on day 6. No differences in mortality were observed between the two sub-groups and they were evaluated as one entity. No differences in mortality were present

between rats treated with convalescent burn serum and with normal
serum, until the 14th day after burn when mortalities were 24 and
48 percent, respectively (p less than 0. 05). At 3 weeks mortali-
ties were 48 and 71 percent (p less than 0. 05) and at 5 weeks 55
and 81 percent, respectively (p less than 0. 05). At termination of
this experiment 13 weeks post-burn, the mortalities of the two
groups were 93 and 97 percent, respectively.

    7.   In experiment III, test-burns were identical with those
in experiment II. Six types of sterile sera were evaluated in a
total of 40 rats for each serum in three series of experimental
burns, done one week apart under randomized conditions. The
six types of sera were:

        A.   Convalescent serum, collected 3 months after 1
            burn (25% body surface).

        B.   Normal serum from unburned rats.

        C.   Serum from unburned rats, given 2 intramuscular
            injections of 0. 3 ml. of sterile complete Freund's
            adjuvant 15 days apart and bled 1 month after the
            first injection.

        D.   Burn serum collected 1 month after the first and
            10 days after the last of 5 burns (5% body surface

each) inflicted 5 days apart; donors housed at

Walter Reed.

E.   Same as Group D; donor animals burned and kept at

Georgetown University.

F.   Same as Group D and E; donor animals given intra-

muscular injections of Freund's adjuvant as in

Group C, with the first and the fourth burn (days 1

and 15); housed at Georgetown University.

The first dose of serum (0. 5% body weight) was given intraperitoneally

immediately after burn.   The second dose (0. 4% body weight) was in-

jected 24 hours, and the third dose (0. 3% body weight) 48 hours,

following burn, both subcutaneously in unburned areas.   Later in-

jections (0. 2% body weight) were administered intramuscularly on

days 3, 6, 10, 13, 17, 20, 24, 27, 31, 38, 45, 52, and 59 following

burn.   The total dose during the first week amounted to 1. 4% of

body weight (or for comparison: 980 ml/ 70 kg. man), and the total

dose in animals which survived on day 59 was 3. 6% body weight

(or 2520 ml/ 70 kg. man).

As in Experiment II, the survival trend was consistently in favor of

the animals treated with 3-month burn convalescent serum.   The

differences in mortality between convalescent and normal serum groups (A and B) were significant at 2, 4 and 5 weeks following burn (p less than 0.05). Convalescent serum could not prevent a steady rise in mortality after the 5th week; when serum treatment was discontinued at 2 months, mortality in groups A and B was similar (55% and 60%, respectively). No significant differences in mortalities were observed at any time after burn between rats treated with normal serum from unburned donors versus serum from unburned donors injected twice with complete Freund's adjuvant. (Group B and C). Likewise, almost identical results were obtained with sera from repeatedly burned donors (Group D and E), regardless of location of housing (Georgetown University versus Walter Reed Army Institute of Research). Injections of Freund's adjuvant (Group F) did not alter the properties of the serum in a way that influenced the survival of burned and treated animals.

Treatment with one-month convalescent burn serum increased mortality on days 2, 3 and 4 after burn when compared with normal serum treatment. (p less than 0.05, 0.01 and 0.05, respectively).

    8.   Experiment IV was a randomized, double-blind study of the effect of three types of sterile sera: 3-month burn convalescent serum, 3-month serum from skin-excised rats, and normal

control serum. Serum dosage was as in Experiment III. Seven consecutive series comprised a total of 115, 114, and 115 animals, respectively, in the three treatment groups.

The first four series of test-burns of 55-65% surface area had a higher 48-hour mortality than in previous comparable experiments. This was ascribed to a lower relative room air humidity (30-40%) as compared to a relative humidity of 70-80% in previous experiments. Under these conditions of high initial mortality from burn shock, aggravated by evaporative loss of water through burned skin, there were no significant differences in mortalities at any time during 30 days after burn between groups treated with 3-month convalescent serum and normal serum. The group treated with serum from skin-excised rats had significantly higher 48-hour mortalities than the normal serum group (p less than .02).

When the burn area was reduced to 40-50% skin surface in the last 3 series of this experiment, the 48-hour mortalities were halved, but again no effect of convalescent burn serum was seen on either 48-hour mortality or mortalities at any subsequent time up to 30 days following burn. However, analysis of the effect of convalescent burn serum versus normal serum, based on animals surviving 48

hours after a 55-65% burn, showed a significant effect of convalescent serum on mortality at 4 days (36% versus 59%) and at 7 days (40% versus 59%), p less than 0.05 in both comparisons.

Similar analysis of rats burned 40-50% showed no differences between treatment groups.

By pooling all animals from experiments II, III, and IV burned 55-65% surface area on a 48-hour survival basis, the effect on mortality of convalescent serum versus normal serum became highly significant at 4 days (15.5% versus 30.5%, p less than 0.01), at 7 days (24.4 versus 38.9%, p less than 0.01), at 10 days (28.8 versus 45.2%, p less than 0.01) and at 14 days (30 versus 48.4%, p less than 0.005).

    9.   CONCLUSIONS.   Treatment with sterile homologous burn convalescent serum resulted in a moderate but significant lowering of the mortality of very severely burned rats (55-65% surface area, full-thickness burn at 260° C.).   This serum had no effect on survival in burns of less than 50% surface area.   Serum from rats convalescing from infected wounds following surgical excision of skin had no protective effect in severe burns.

The present experiments provide only indirect evidence in support of the toxin-theory of "toxemia" and delayed death in burns.

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TABLE I

CUMULATIVE MORTALITY PER CENT

Rats burned 50% full-thickness (480° F)
Sterile serum and dextran/saline injected as indicated.

Day	Group A n = 10	Group B n = 20	Group C n = 15	Group D n = 100	Injection Day of Burn Only
1	0	0	7	5	All four groups
2	11	30	13	40	injected intra-
3	16	70	20	53	peritoneally im-
4	"	80	27	67	mediately after
5	2?	?0	53	84	burn.
6	"	"	60	89	
7	"	"	67	90	3% body weight
8	"	95	??	92	of colloid/
9	"	"	"	93	saline
10	"	"	"	95	solution.
11	"	100	"	97	
12	26		100	100	
13	"				
14	84				
16	"				
17	89				
18	"				
20	100				

Group A: Intraperitoneal injection of sterile convalescent serum from rats burned 25-30% body surface (full-thickness) 2 months previously. Dose 1.5% body weight of serum plus 1.5% body weight of 6% dextran in saline.

Group B: Intraperitoneal injection of sterile normal serum from unburned rats. Same dose of serum and dextran/saline as for Group A.

Group C: Intraperitoneal injection of sterile "wound serum", obtained from rats which had 25-30% skin area excised 2 months previously. Same dose of serum and dextran/saline as for groups A and B.

Group D: Intraperitoneal injection of 3% body weight of dextran/saline.

TABLE II

CUMULATIVE MORTALITY PER CENT

Rats burned 60-65% full-thickness (500°F)
Subcutaneous injections of 0.9% NaCl - solution (14% body weight)
Sterile serum injected as indicated.

Day	Serum cumul. dose % body weight	Group A n = 29	Group B n = 31	Significance p
1		0	0	
2		3.4	6.5	
3		6.9	9.7	
4		"	16.1	
5		10.3	22.5	
6		20.7	25.8	
Week				
1	1.6	20.7	29.0	< 0.05
2		24.1	48.4	
3		48.3	71.0	< 0.05
4		55.2	74.2	
5		"	80.6	< 0.05
6		62.1	83.9	
7		72.4	"	
8		82.8	"	
9		86.2	"	
10		"	96.8	
11		93.1	"	
12		"	"	
13		"	"	

Group A: 5-month convalescent serum, one burn, 25% surface area.
Group B: Normal serum (unburned rats).

One-half of each group received injection of serum in a dose of 1% body
weight immediately following burn, 0.3% body weight on days 4 and 7.
Total serum dose: 1.6% body weight.

The other half of each group received one injection of 1% body weight on
day 2 and 0.6% body weight on day 6. Total serum dose: 1.6% body
weight.

TABLE III

CUMULATIVE MORTALITY PER CENT

Rats burned 60-65% full-thickness (500°F)
Subcutaneous injections of 0.9% NaCl solution (14% body weight)
Sterile serum injected as indicated.

Day	Serum cumulative dose % body wt.	Group A n = 40	Group B n = 40	Group C n = 38	Group D n = 40	Group E n = 40	Group F n = 40	Groups D, E, F pooled n = 120
1	0.5	0	2.5	13.2	2.5	5.0	5.0	4.2
2	0.9	10.0	15.0	23.7	30.0	25.0	30.0	28.3
3	1.2	17.5	22.5	39.5	47.5	40.0	45.0	44.2
4	"	22.5	32.5	42.1	"	47.5	47.5	47.5
5	"	25.0	40.0	44.7	"	"	"	"
6	1.4	"	"	"	"	"	"	"
Week								
1	1.4	27.5	42.5	44.7	47.5	47.5	47.5	47.5
2	1.8	"	45.0	"	"	50.0	50.0	49.2
3	2.2	32.5	"	"	"	"	"	"
4	2.6	"	50.0	"	"	"	"	"
5	2.8	35.0	57.5	"	"	"	"	"
6	3.0	42.5	60.0	"	50.0	52.5	55.0	52.5
7	3.2	47.5	"	50.0	62.5	62.5	67.5	64.2
8	3.4	55.0	"	55.3	65.0	70.0	70.0	68.3
9	3.6	"	"	57.9	"	"	"	"
10	"	"	"	60.5	70.0	"	"	70.0
11	"	57.5	"	"	72.5	"	"	70.8
12	"	60.0	65.0	"	"	75.0	72.5	73.3
13	"	"	"	"	"	80.0	"	75.0
14	"	"	67.5	63.2	"	"	"	"

Group A: 3-month convalescent serum, one burn 25% surface area.
Group B: Normal serum (unburned rats).
Group C: Serum from unburned rats; injections of complete Freund's adjuvant intramuscularly, 15 days apart; serum harvested 1 month after 1st injection.
Group D: 1-month   serum, 5 burns (total 25% surface area); serum collected 1 month after first burn; location, Walter Reed.
Group E: Same as D; location, Georgetown University.
Group F: Same as E; injections of complete Freund's adjuvant 15 days apart (with first and fourth burn).

TABLE IV

COMBINED DATA FROM SERIES 1959-1961

CUMULATIVE MORTALITY PER CENT

A. All rats, burns ranging from 40 to 65 per cent surface area.

Time after burn	Convalescent burn serum	Normal serum	p-value of difference in mortality
	n = 184	n = 185	
2 days	34. 2	31. 3	not significant
4 days	50. 0	55. 1	0. 05
7 days	54. 3	60. 5	0. 05
10 days	57. 0	63. 7	0. 04
14 days	57. 6	65. 4	0. 03

B. Rats surviving 48 hours after burns ranging from 40 to 65 per cent S. A.

	n = 121	n = 127	
4 days	23. 9	34. 6	0. 02
7 days	31. 4	42. 5	0. 02
10 days	34. 7	47. 2	0. 01
14 days	35. 5	49. 6	0. 008

C. Rats surviving 48 hours after burns ranging from 55 to 65 per cent S. A.

	n = 90	n = 95	
4 days	15. 5	30. 5	0. 01
7 days	24. 4	38. 9	0. 01
10 days	28. 8	45. 2	0. 01
14 days	30. 0	48. 4	0. 005

TABLE V

NEW SERIES 1960-1961, COMPARING BURN CONVALESCENT SERUM, CONVALESCENT SERUM FROM RATS WITH OPEN WOUNDS, AND NORMAL SERUM.

CUMULATIVE MORTALITY PER CENT

A. All rats, burns ranging from 40 to 65 per cent surface area.

Time after burn	Convalescent burn serum	Normal serum	Conv. serum, open wounds	p-values Burn Conv. vs. normal	p-values Open Wound conv. vs. nor.
	n = 115	n = 115	n = 114		
2 days	51. 3	44. 3	57. 0	not. signif.	0. 02
4 days	72. 1	73. 9	80. 7	"	not signif.
7 days	73. 0	75. 6	81. 5	"	"
10 days	74. 7	75. 6	82. 4	"	"
14 days	75. 6	77. 3	82. 4	"	"

B. Rats surviving 48 hours after burns ranging from 55 to 65 per cent S. A.

	n = 25	n = 32	n = 26		
4 days	36. 0	59. 3	50. 0	0. 05	not signif.
7 days	40. 0	59. 3	50. 0	0. 05	"
10 days	44. 0	59. 3	50. 0	not signif.	"
14 days	48. 0	65. 6	50. 0	"	"

C. Rats surviving 48 hours after burns ranging from 40 to 50 per cent S. A.

	n = 31	n = 32	n = 23		
4 days	48. 3	46. 8	60. 8	not signif.	not signif.
7 days	48. 3	53. 1	65. 2	"	"
10 days	51. 6	53. 1	69. 5	"	"
14 days	51. 6	53. 1	69. 5	"	"

PROGRESS   REPORT

JANUARY 1962

EVALUATION OF THE "TOXIN ANTI-TOXIN" EFFECTS

OF SERUM FROM BURNED INDIVIDUALS

ONR PROJECT NO. 774

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CLERICAL STAFF
SUPPORT OF TISSUE
GRAFT REGISTRY

EVALUATION OF THE "TOXIN ANTI-TOXIN" EFFECTS
OF SERUM FROM BURNED INDIVIDUALS

The Tissue Bank of the Naval Medical School has continued the
investigation of "toxins" in the serum of burned patients. The
obvious importance of the toxin anti-toxin concept in burns with
its implication regarding the use of convalescent burn serum in
the treatment of the acutely burned patient, necessitates a con-
tinuing effort to evaluate existing tests for the detection of such
toxins.

In review it must be remembered that the modern revival of in-
terest in the burn toxin field resulted from the treatment of several
children with convalescent burn plasma from the Chicago school
fire in December of 1958. · Some of the observers at that time,
particularly Dr. Sol Roy Rosenthal and Dr. Hartney, were led to
the conclusion that convalescent burn plasma had beneficial effects
demonstrated by reduced fever, increased urinary output, and
general over-all well being of the children. Others including
Dr. Callahan, Chief of Surgery at the hospital in which the children
were treated, thought the clinical data was insufficient to permit
valid judgment.

In general our work is divided into three categories.

I. Tissue Culture Evaluation of the cytotoxic effect of burn sera. This has occupied approximately 70% of our time.

II. Evaluation of the _in vivo_ physiologic effects in animals caused by the administration of burn serum or blood.

III. The investigation of new antigenic substances in burn serum by means of _in vitro_ immunologic procedures, such as the Schultz-Dale guinea pig smooth muscle anaphylaxis phenomena.

I.   Tissue Culture

   A.   Method

      The details of the tissue culture technique which has been used in the evaluation of the cytotoxic effect of burn serum, were outlined in our previous report of January, 1961.   In general the test consists of the application of 20% test sera to freshly planted HeLa cultures with subsequent attempts to evaluate the outgrowth of these cells by means of a visual and subjective observation.   The technique duplicates as closely as possible the technique that was originally used in the spring of 1959 in the testing of the serum from the children in the Chicago school fire.   At that time the burn patient's sera demonstrated a toxic effect which could be counteracted with convalescent burn blood at a level 3.2 times better than was the counteractive effect of normal blood.

B. Source of Material

(1) Serum was collected throughout the Navy from patients with over 10 per cent second and third degree burns. In addition, several civilian collaborators including Professor Dogo of Padua, Italy, Dr. Samuel Fogelson of Cook County Hospital, Chicago, Illinois and Dr. Strong of the Washington Hospital Center, Washington, D. C., provided blood samples from acutely burned patients.

(2) A separate double blind study was carried out with specimens being provided by Dr. N. Georgiade of Duke University, Durham, North Carolina, Dr. Hagerty of the Medical College of South Carolina, and Dr. T. G. Blocker of Galveston, Texas. Blood specimens were obtained from acute and convalescent burn patients, as well as normal controls. An identification card bearing all data pertinent to the source of each sera was sealed in an envelope and not opened until all testing had been completed. The specimens were drawn into non-toxic vacu-container test tubes. The serum and cells were separated and both were then sent air mail to the Tissue Bank of the Naval Medical School. On arrival, the specimens were treated in one of three ways. A portion of the serum was immediately frozen. Another aliquot of the serum was replaced over the patient's own cells and allowed to remain in the refrigerator for a

period of two weeks prior to separation and storage of the serum in a frozen state. Finally, a pool of normal AB positive serum was overlayed over a portion of the patient's clot and allowed to remain in contact for a period of two weeks at 4° C. prior to separation and storage of the AB serum at freezer temperatures.

(3) Blood was collected from dogs before and after burning, the collection and burning procedures were carried out at the Naval Medical Field Research Laboratory, Physiology Branch, Camp Lejeune, North Carolina, under the direction of Captain G. L. Calvy.

C. Results

As can be seen in Table I, normal sera of both adults and children displays no significant toxicity when either frozen or kept in a refrigerator for two weeks prior to separation and freezing. It must be pointed out that these specimens are collected in the laboratory and handled and prepared immediately following the formation of clot. The only toxic specimen was obtained from an adult female who was menstruating.

In Table II it can be seen that both acute and convalescent sera from burned patients collected as frozen specimens, demonstrate virtually no toxicity by the tissue culture method. One frozen

TABLE I

CONTROL SERA FOR HeLa CELL TEST

		Adult Male	Adult Female	Child Male	Child Female	Total
Frozen						
	n	64	1	16	7	88
	t		1*			1
Refrig. with clot						
	n	9	2			11

t - toxic   *during menstruation
n - non-toxic or normal

TABLE II

RESULTS OF HeLa CELL TESTS
OF HUMAN BURN SERUM

ACUTE

		Frozen	Refrig. clot	Refrig.	Refrig. AB
Adult Male					
	t		1		
	pt				
	n	21	4	2	1
Adult Female					
	t	1			
	pt				
	n	5	4	3	1
Child Male					
	t				
	pt		1		
	n	1		1	
Child Female					
	t				
	pt				
	n	1		1	

CONVALESCENT

		Frozen	Refrig. clot	Refrig.	Refrig. AB
Adult Male					
	t				
	pt		1		
	n	10		2	
Adult Female					
	t				
	pt				
	n	1			

t - toxic
pt - partially toxic
n - non-toxic

specimen collected 6 days·after 80% burn in an adult female, was

toxic. However, this specimen was collected after death and it

was subsequently noted that the patient died of septicemia.

Two specimens which proved to be toxic when stored

as whole blood in the refrigerator prior to testing, had arrived

here from other hospitals and had been exposed to some degree of

room temperature incubation of 18 hours or more prior to

refrigeration. Also the fact that a convalescent specimen, treated

in the same manner, developed partial toxicity would tend to rule

out a primary cytotoxic effect.

Therefore, the data collected so far would appear to

demonstrate a lack of toxic effect by the HeLa test, if the serum

is not subjected to unusual storage conditions.

Table III records the results of the double blind study

of burn and normal specimens. The differentiation between acute

and convalescent burn sera is most difficult. It has been suggested

that the toxic antigenic material should be present only for a few

days or at most a week or-ten days following the burn. After this

time the appearance of a high titre of antibodies would probably

eliminate antigens circulating in the serum. However, the

theoretical concept of others working in this field would suggest

that as long as the wounds of the patient are open, toxins are newly formed and continually released·into the blood stream. Therefore, the time of "acute toxicity" is open to question. Due to the fact that positive toxic tests were obtained as long as 15 days following burns when the HeLa cell test was originally used for the detection of burn toxins, it would seem reasonable that a period of at least 15 days could be included in the acute period. However, the fact that the eschar from burns rarely starts sloughing until about this time and the wound is usually open until 30 days in significant third degree burns, it would then be feasible to extend the "acute burn period" to a 30 day interval. While the selection of this time period may result in criticism, the actual evaluation of individual specimens and a tabulation of these results is available for interpretation.

From these tests it is quite clear that the acute burn serum (by our definition) when separated immediately from its clot and shipped at room temperature to our laboratories and with subsequent freezing, demonstrates little toxicity. The convalescent burn specimens demonstrate a similar percent toxicity. The meaning of this is not clear and there seems to be no definite relationship between sex, age, nor extent of burn of the patient, and the apparent toxicity of these specimens. In view of the recent serological evi-

TABLE III a

RESULTS OF HeLa CELL TESTS OF HUMAN BURN SERUM

		Acute			Convalescent		
		Froz.	Clot	AB	Froz.	Clot	AB
Adult Male							
	t		2	3	1		1
	pt	1	1		1		
	n	7	4	2	3		
Adult Female							
	t		1	5		5	2
	pt		2	1		1	1
	n	4	2		7	3	6
Child Male							
	t				1	1	
	pt		1	1			
	n	4	1	1	2	1	2
Child Female							
	t	2	1	3	2	3	2
	pt				1	1	2
	n	1			6	2	3

		· Control		
		Froz.	Clot	AB
Adult Male				
	t	1		
	pt			1
	n	1	1	
Adult Female				
	t	1		
	pt	1		
	n			

t   - toxic
pt - partially toxic
n   - non-toxic

Sources of Unknown Burn Sera Samples.

R. F. Hagerty   N. Georgiade
9   8
T. G. Blocker
46

TABLE III b

SUMMARY OF HeLa CELL TESTS

A. Frozen sera from special blind study

	Acute	Convalescent
t	2	4
pt	1	2
n	16	18

B. Frozen sera from general collection

	Acute	Convalescent
t	1 (co tami ated)	0
pt	0	0
n	28	11

C. Grand total all HeLa cell tests

	Acute	Convalescent
t	3	4
pt	1	2
n	44	29

t - toxic
pt - partially toxic
n - normal

dence of the development of antibodies in convalescent burn serum against autologous skin, it might be logical to assume that HeLa cells that are epithelial in origin may in some manner suffer from the antibody effect of convalescent serum. On the other hand such cellular antibodies are not known to be effective against cells in the absence of complement. All specimens used in the present experimentation were decomplemented at 56° C. for 30 minutes. The high degree of toxicity in the small number of "control" specimens also would shed doubt on the validity of this test technique using specimens transported long distances.

It is further noted in Table III that serum when allowed to remain for two weeks in the refrigerator with the clot, a high percentage of the specimens become toxic, whether the serum originated from the burn patient or whether the serum was obtained from a normal pool of AB serum. Work done in our laboratory and in the Tissue Culture laboratories at the Naval Medical Research Unit #4 at Great Lakes would indicate that the storage of whole blood at room temperature in excess of 48 hours results in the regular production of a cytotoxic activity in the serum. The clot specimens sent from the various collaborators traveled one to two days in transit between sender and recipient, and therefore, suffered room

temperature incubation. Also the serum was not completely removed from the cells by ordinary centrifugation. Therefore, it would appear that the toxicity that was thus demonstrated was merely the normal development of toxicity which can be seen with any specimen allowed to remain at room temperature for the period noted. It is further speculated that this toxicity may well be the result of enzymatic degradation of proteins in the normal blood sample at room temperatures. Because of the known elevation of peptidases as well as other enzymes in burn serum, it is theoretically possible that the degradation of proteins could proceed at a more rapid rate in the burn specimen. This might explain the partial toxicity which we originally observed in burn blood allowed to remain for two weeks at refrigerator temperature prior to separation of serum.

Table IV demonstrates the development of the normal toxicity in serum when stored at room temperature.

We feel that we are unable to utilize this testing technique to evaluate this so-called cytotoxic effect of burn serum, and thus are unable also to evaluate the beneficial effect of convalescent serum.

D.   Other tissue culture tests utilized in an attempt to detect the toxic factors in burn skin or serum.

TABLE IV

HeLa CELL GROWTH IN NORMAL SERA
EFFECTS OF EXPOSURE OF BLOOD TO ROOM TEMPERATURE
STORAGE

Time of Storage prior to Separation of Serum	Average Growth After 4 days
0 hours	4 plus
24 hours	4 plus
48 hours	2-3 plus
72 hours	1 plus
96 hours	1 plus
Refrigerated 96 hours	4 plus
Fresh Frozen	4 plus

1. Freshly planted cultures of trypsinized guinea pig kidney were exposed to one to three dilutions of homogenized guinea pig skin extract. As can be seen in Table V, burned and unburned skin homogenized extracts proved to be toxic before the particulate matter was removed by centrifugation. But both were non-toxic in the clear centrifuged samples.

2. In order to determine if another cell line might be more sensitive to the effects of the toxin in burn sera, 10 cell lines available in our laboratories were subjected to 20% concentrations of sera taken from significantly burned patients with 10% or more burns. See Table VI. In only one of the ten was there a definite toxic effect observed. This was in Strain 929 mouse fibroblast. It was noted that normal serum demonstrated the same toxic effect in this particular tissue culture cell. A beneficial effect on growth was observed in the 2544 cloned human skin strain, and 3526 cloned monkey kidney on chemically defined media. However, these same beneficial effects could be obtained with 20% normal serum. It was, therefore, concluded that no cell line in the laboratory offered any greater sensitivity for the toxic effect of burn serum.

3. Fresh kidney trypsinized test tube cultures were exposed to dog serum obtained before and at regular intervals

following burning. In 128 cultures there was no evidence of toxicity with any of the dog serum samples so tested.

4. A measurement of the viability of cells by means of the decolorization of methylene blue in serially diluted agar tissue culture slants was attempted.

In Table VII it can be seen that serial dilutions of HeLa cells in the presence of normal or acute human burn serum (17 days after burning) revealed no toxic action of the burn material. In fact in this single case it would appear that the burn serum had a somewhat more beneficial effect than the normal serum in promoting the growth of cells as measured by this testing technique. In the second portion of the table is a description of the results obtained when tissue cultures containing a primary trypsinized culture of dog kidney cells were exposed to pre- and post-burn specimens from another dog receiving 30% third degree burns. As can be seen, particularly with the cell concentration of 625,000, there was no significant difference in the decolorization time between the pre-burn, immediate post-burn or convalescent burn serum specimens.

5. In an attempt to determine if the cell concentration of the test tube HeLa cultures had any influence on the development of toxic activity, a series of cultures was set up with varying quan-

TABLE V

EFFECTS OF SALINE EXTRACTS OF BURNED SKIN ON GUINEA PIG PRIMARY KIDNEY TISSUE CULTURES

	Crude	Centrifuged
Unburned skin	toxic	no effect
Burned	toxic	no effect

TABLE VI

CELL LINES USED IN AN ATTEMPT
TO DETECT "BURN TOXIN"

2071	Mouse "L"	Chemically defined media
3206	Monkey Kidney	Chemically defined media
2544	Cloned human skin	
2806	Human skin	
686	HeLa	
1469	Mouse liver	
929	Mouse fibroblast	
457	Human skin	
3526	Cloned Monkey kidney	Chemically defined media
721	Mouse liver	

TABLE VII

DEHYDROGENASE ACTIVITY MEASURED IN HeLa CELLS

Cell counts x 10^4 per ml.

	500	250	125	62.5	31.2	15.6
Saline	0.13	0.25	1.00	2.16	4.75	-10
Normal human serum	0.16	0.41	0.78	2.16	8.33	-
Acute burn serum 17 days	0.16	0.16	0.75	1.00	3.33	3.83

Expressed as Fractions of Hours until First Discernible Decolorization

DEHYDROGENASE ACTIVITY MEASURED IN PRIMARY DOG KIDNEY CELLS

Cell counts x 10^4 per ml.

	500	250	125	62.5	31.2	15.6
Saline	0.16	1.25	3.25	7.6	-	-
Pre burn	0.16	1.25	1.41	2.75	4.91	7.6
10 min.	0.16	0.33	1.41	2.75	7.66	18.16
24 hours	0.16	0.33	1.41	2.75	7.66	-
6 days	0.16	0.33	1.25	2.75	4.41	-
34 days	0.16	0.33	1.41	3.25	-	-

Sera from a single dog receiving 30% 3rd degree burn

TABLE VIII

THE EFFECT OF CELL CONCENTRATION
ON "TOXICITY" OF BURN SERUM IN HeLa CULTURES

20% Serum	No. Cell per Culture 0.5 ml.	Evaluation
192	200,000	n
10	200,000	n
192	100,000	n
10	100,000	Lost
192	50,000	n
10	50,000	n
192	25,000	pt
10	25,000	pt
192	12,500	pt
10	12,500	pt
192	6,250	pt
10	6,250	pt
192	3,125	t
10	3,125	t

192 – Normal O positive pool
10 – 6 days after 45% 3rd degree burn
    32 year old male

n – normal
pt – partially
    toxic
t – toxic

tities of cells in the presence of either normal 0 + human serum or serum taken 6 days after a 45% third degree burn in an adult male. No differential reaction is noted. It was possible, however, to equate cell concentrations of 50, 000 or more per . 5 ml. culture with outgrowth of 4 + full sheets of cells. Less than optimal growth occurred with 25, 000 cells. See Table VIII. This would seem to indicate that the 32, 000 cells per . 5 ml. culture used in the routine HeLa tests may lie on the border line of optimal and beginning sub-optimal growth patterns.

SUMMARY AND DISCUSSION

Table IX contains a list of various tissue culture techniques and procedures which were used in an attempt to identify the cytotoxic activity of serum obtained from burned patients. None of the techniques mentioned here were useful in the detection of such a toxic effect. The only hint of toxicity would seem to show a relationship between some time interval of room temperature storage of the whole blood specimen prior to the development of its toxic effect.

The following observations might then be made.

a. In order to evaluate accurately the toxic potential of

TABLE IX

SUMMARY
TISSUE CULTURE TESTS

Tissue	Test Agent
HeLa	Human sera
10 stock cell lines	Human sera
Dog kidney	Dog sera
Guinea pig kidney	Guinea pig skin extracts
HeLa (dehydrogenase)	Human sera
Dog kidney (dehydrogenase)	Dog sera

blood specimens, the nature of transportation and storage procedures must be clearly outlined and considered in reference to the final tissue culture evaluation.

   b. In our present series it is regrettably noted that only a few children were included in the acute burn series. Originally the tissue culture test had utilized sera from a group of children burned in a school fire. Therefore, complete correlation between our results and the original results would not appear to be possible. The pathophysiology of the process by which skin or surface epithelial proteins are degradated to become antigenic toxins would not seem to be related directly to the age of the patient. Further if such an interpretation were made this would in fact remove adults from consideration of the proposed·burn toxin theory.

   c. The interesting retrospective discovery of cytotoxicity obtained from a normal but menstruating woman, would indicate that such factors must also be taken into account. In general it would appear that all aspects of the normal development of cytotoxicity whether from storage or normally produced in various physiologic phases of cyclic metabolic activity in the human system must be evaluated prior to a conclusive or even indicative study of burn cytotoxicity.

d. Recently it has been reported that burn sera can be shown to have a cytotoxic action in tissue culture when measured as inhibition of outgrowth or migration of cells from a fresh explant of bone marrow. This phenomenon seems to be related to a similar depressant effect on the motility or outgrowth of cells noted in spleen explants taken from animals or man in whom a known hypersensitivity against such substances such as PPD are present, and then grown in the presence of minute traces of this PPD antigenic material. The use of such motility inhibition testing techniques may prove to be a sensitive indicator of the proposed "toxin" in the serum of burn patients.

II. In Vivo Measurement of the Physiologic Effects of Burn Products from Burned Individuals.

Serum taken from dogs prior to burning and at regular intervals after burning beginning at 10 minutes up to 6 days after burn, were injected in quantities of .1, .2, .3, 1.0 cc. intraperitoneally into an inbred strain of Balb C mice. No toxicity was noted.

In order to determine if preconditioning of the animal by means of some toxic agent might predispose it to the mortal effects of a burn toxin substance, three groups of tests were performed.

1. A group of 48 F 1 hybrid mice (resulting from a cross of Balb C and C3H inbred strains) were exposed to radiation in the amount of 800r given by a cobalt irradiator (LD 0 to 5). Burn sera was taken from dogs receiving 30% third degree burns by means of infrared radiation at Camp Lejeune Medical Field Research Laboratory. Approximately 8 days following the administration of radiation, a time when animals are most susceptible to any exposure because of extreme depletion of the lymphocytic cells of the immune system, 12 mice were injected with .2 cc. of saline, 12 mice injected with .2 cc. of serum obtained one hour after burning, 12 mice injected with 24 hour post burn serum, and 12 mice given no injection. Two mice died immediately after injection and were, therefore, discounted from the study. All other animals survived for a 24 day period. It was thus felt that radiation was not a satisfactory predisposing insult to mice in preparation of them as test animals for burn toxic sera.
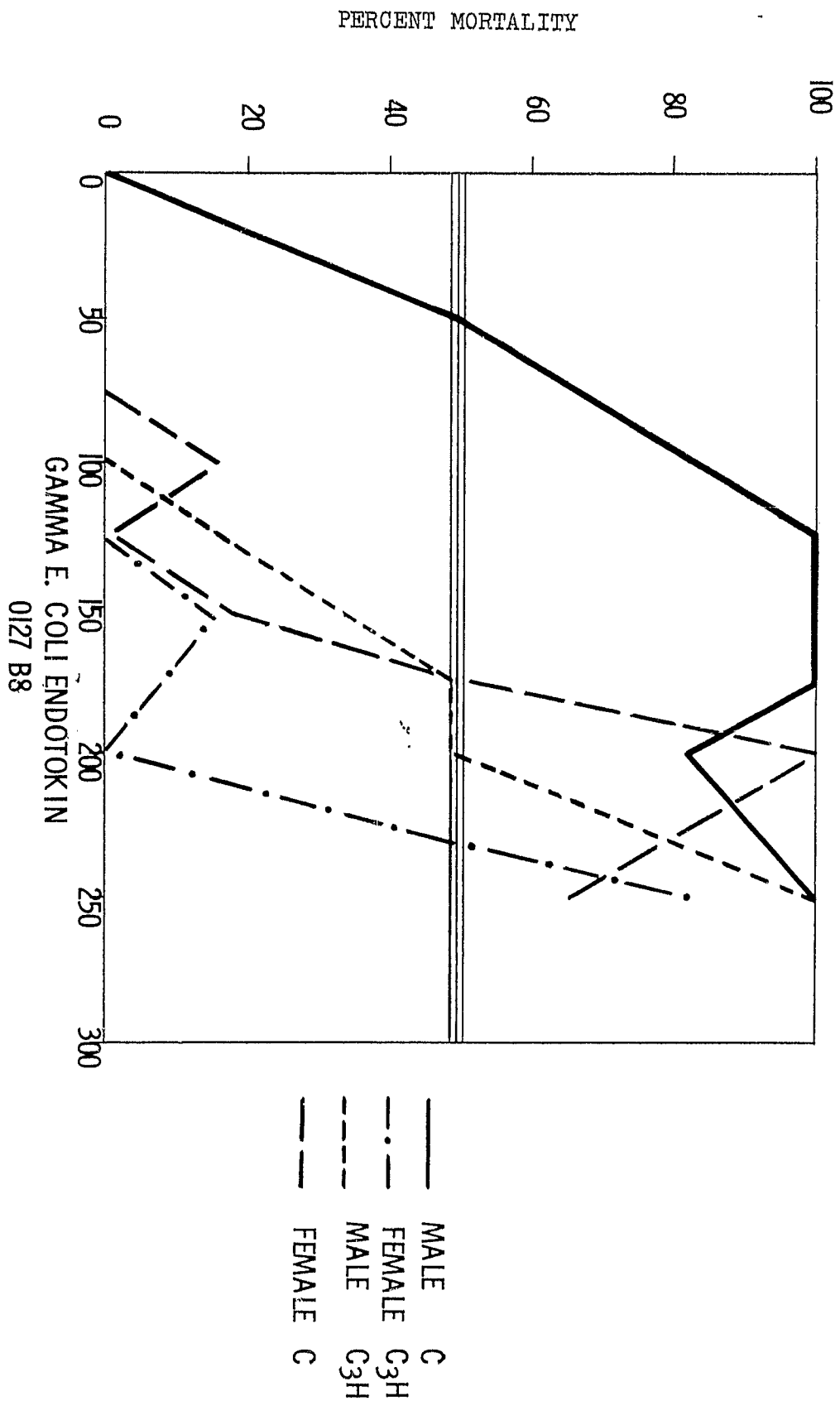
2. Another experiment was carried out in which F 1 hybrids of Balb C and C3H mice were irradiated at 500r by a cobalt 60 source. The group consisted of 12 animals which were irradiated and then one-half of the group was injected with isologous normal whole blood. The other half was injected with isolo-

gous whole blood from animals burned at 70° for 10 seconds over two-thirds the body surface. The blood was collected 5 minutes post-burning. All 12 animals in this group survived. The blood in this case was injected intravenously and very slowly. Apparently blood is tolerated intravenously much better than intraperitoneally for quantities of . 7-. 8 cc. were given with no difficulty. However, it was noted in previous experiments that rapid injection of even quantities of . 2-. 3 cc. caused sudden death in mice.

      3. An attempt was made to predispose mice to burn toxic sera and burn blood by means of graded doses of bacterial lipopolysaccharides in the form of the endotoxin of the E. Coli -127-B8. Lethal dose curves for this substance were obtained in groups of 96 mice with separation of the two inbred strains, Balb C and C3H, with further segregation by means of sex. See Graph I. It was noted that the most sensitive mouse was the male Balb C which was susceptible to an LD 50 dose of approximately 50 gamma of E. Coli endotoxin. The female Balb C and the male C3H each exhibited more resistance to the endotoxin with an LD 50 dose of approximately 175 gamma. The most resistant of the animals was the female C3H mouse which exhibited an LD 50 dose of approximately 230. gamma. It was further noted that Proferrin, a saccharated iron

GRAPH I

LETHAL DOSE CURVES FOR E. COLI 0127.B8 (Ditco)
IN SEX AND STRAIN SPECIFIC MICE

PERCENT MORTALITY

GAMMA E. COLI ENDOTOKIN
0127 B8

MALE C
FEMALE C₃H
MALE C₃H
FEMALE C

190

compound given in a quantity of 0. 2 cc. intraperitoneally two hours prior to the administration of endotoxin greatly potentiated the lethal effects of endotoxin. This has been explained as a result of blockade of the reticuloendothelial system.

Table X presents the data resulting from the combined insult of blood, either burn or normal, with endotoxin. There is no significant difference in the mortality observed when isologous normal blood from the same strain of mice is administered prior to endotoxin as compared to isologous burn blood from a similar burn source. The animals in this instance were burned for 15 seconds at 70° C. in scalding water over two-thirds of the body surface. Blood was collected five minutes after the burn.

The heterologous administration of guinea pig normal blood and guinea pig burned blood also failed to demonstrate a difference in mortality. However, it was noted that the mortality which occurred with the administration of either burn or normal blood of both the isologous and heterologous source, prior to the injection of endotoxin, caused a mortality which is greater than when endotoxin is used alone. The increase in mortality was similar to that noted when Proferrin was given prior to the administration of the endotoxin.

TABLE X

MORTALITY IN MICE FROM ENDOTOXIN + BURN BLOOD PRODUCTS

			125 gamma	50 gamma	25 gamma	12.5 gamma
Male C	Endotoxin alone		5/6	1/6	0/7	0/6
Male C	Proferrin .2 cc.		4/6	6/6	5/6	2/6
Male C	Isologous burn blood female .2 cc.		6/6	3/6	2/6	2/6
Male C	Isologous normal blood female .2 cc		5/5+	0/3+	1/4+	3/6
Male C	Guinea pig burn blood .1 cc.		5/6	4/6	2/5*	2/6

			250 gamma	200 gamma	100 gamma	50 gamma
Male C3H	Endotoxin alone		6/6	4/6	0/6	0/6
Male C3H	Guinea pig burn blood .1 cc.		5/6	5/5+	4/6	2/6
Male C3H	Guinea pig normal blood .1 cc.		5/5	6/6	5/6	2/6

Immediate (before second injection) deaths + 6/24 with isologous
normal blood

* 2/48 with heterologous
G. P. burn blood

0/24 with Proferrin

0/24 with isologous
burn blood

0/24 with heterologous
G. P. blood

number dead/number animals in each category

This seems to indicate that whole blood, whether from a normal or burned individual, may obstruct the reticuloendothelial system in a manner similar to that of Proferrin.

A number of deaths occurred immediately after the injection of blood prior to the injection of endotoxin. Six of 24 animals receiving isologous normal blood died immediately. None of 30 animals receiving burn isologous blood died. Two of 48 animals receiving heterologous guinea pig burn blood died. One of 30 animals receiving heterologous guinea pig normal blood died. None of 24 animals who received Proferrin alone died. All the blood used in this study was collected in heparinized syringes to prevent hemolysis. It has been suggested by Dr. Feodorov of Russia, that heparin may counteract some of the toxic effects of acute burn serum. Therefore, it would be interesting to repeat this experiment using another method of anti-coagulation.

III. Attempts to isolate antigenic materials in burn serum or blood specimens by means of in vitro immunologic test systems.

Dr. Feodorov at the Hematology Institute in Moscow, Russia, has reported in several papers since 1955, that there is a new antigenic material found in the blood from burned animals

which is not present in animals prior to burning. Furthermore, he

has offered evidence that this new substance is antigenically the

same in all species of animals including goats, humans, dogs,

rabbits and horses. In view of these findings it was considered

that an evaluation of this work might be helpful. Unfortunately

the technique used by the Russian workers to demonstrate these

antigens was not clearly outlined. Apparently guinea pigs were

immunized with burned blood or homogenized burned skin and

subsequently they were desensitized with normal blood or homogen-

ized normal skin from the same species of animal, and finally

challenged with a 1 cc. dose of burned blood intravenously causing

anaphylactic symptoms or death. In one paper describing this work,

155 animals were tested. Blood was taken between 1 and 21 days

post-burn and was demonstrated to contain the new antigen. All

other blood specimens were reported as negative. This 100%

consistency of results in a biologic test system offered the ultimate

mark to shoot for in our repetition of this work. In order to demon-

strate this anaphylactic activity in guinea pigs in a more objective

manner, we decided to utilize the in vitro muscle bath preparation

with a permanent record of the smooth muscle contraction to

sensitizing antigens. This method was chosen also because of its

reported greater sensitivity when compared to the general whole body anaphylactic phenomena.
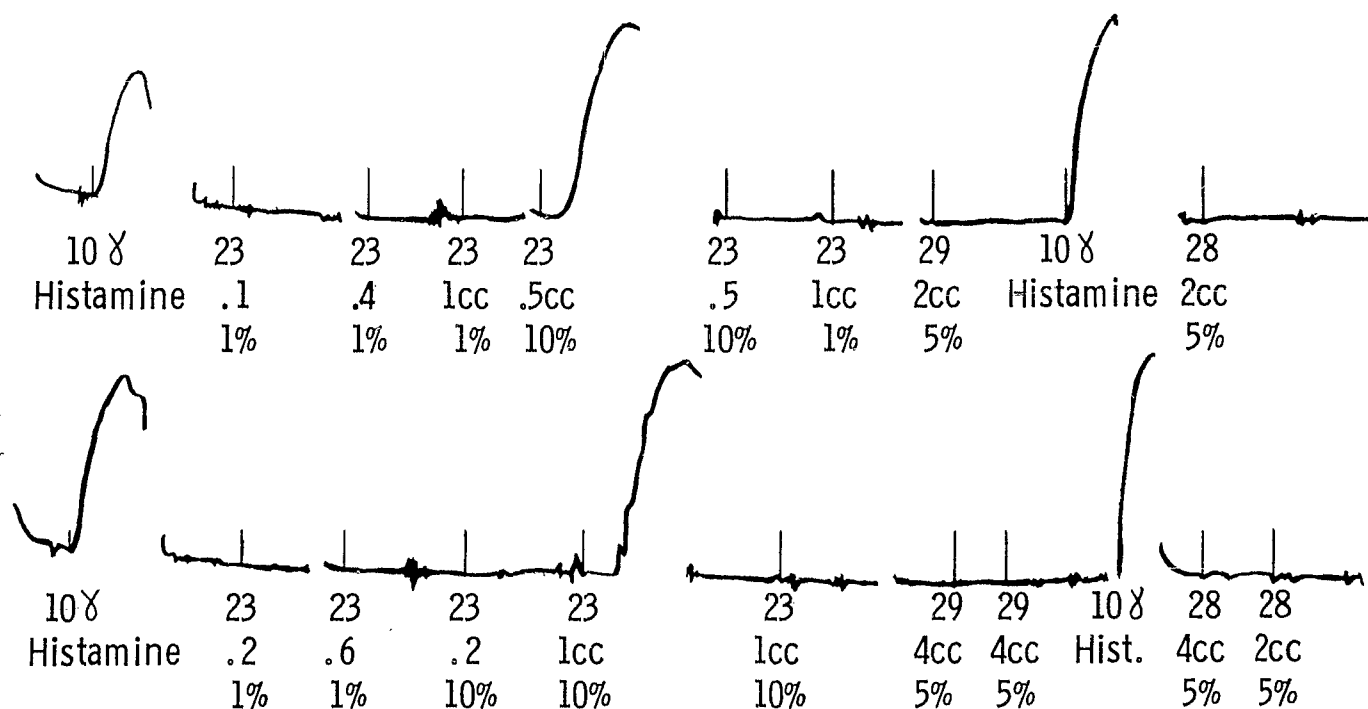
A group of animals were immunized with pooled sera consisting of burned blood-taken from dogs 24 and 48 hours after 30% third degree burns. Guinea pigs were sensitized with a single dose of sera mixed with an equal amount of Freund's adjuvant, injected in fractioned doses in 4 intramuscular sites. After a period of approximately 6 weeks, the animals were sacrificed and used for testing. In Graph II it can be noted that the animals were highly sensitive to normal dog serum in a dose of .05 ml although non-responsive to .004 ml. When desensitized to the normal antigenic material in serum there remained no residual sensitivity to the pool of 24 or 48 hour post-burn serum nor to either of these two sera tested independently. As much as 1/2 cc. of serum was tested in a 50 ml. fluid bath surrounding the guinea pig uterine tissue. Because of the dilution factor it may be possible that the burn toxin antigen may be only weakly antigenic and not demonstrable by this system. Nevertheless, within the limits of the present test system, no new antigens could be demonstrated in the 24 or 38 hours burn specimen.

Another group of guinea pigs were bled by intracardiac

GRAPH II

SCHULTZ-DALE SMOOTH MUSCLE ANAPHYLAXIS

1) Animal sensitized with post-burn sera from a dog (24 and 48 hours)
   sera numbers 28 and 29

2) Preliminary desensitization with normal pre-burn sera from same dog
   serum number 23

          7 Dec 60 - Injected 0.5 ml #28-29 I.M.
          19 Jan 61 - Sacrificed
          G. P. #14

10 ϒ	23	23	23	23		23	23	29	10 ϒ	28
Histamine	.1	.4	1cc	.5cc		.5	1cc	2cc	Histamine	2cc
	1%	1%	1%	10%		10%	1%	5%		5%

10 ϒ	23	23	23	23		23	29	29	10 ϒ	28	28
Histamine	.2	.6	.2	1cc		1cc	4cc	4cc	Hist.	4cc	2cc
	1%	1%	10%	10%		10%	5%	5%		5%	5%

puncture in order to obtain normal samples of sera. Subsequently they were burned with a 30% third degree burn given by an infrared burning apparatus on a shaved, blacked area on their backs and thighs. Following healing, the animals were sacrificed and the smooth muscle which now presumably would contain antibody against the "burn toxin" was then subjected to testing using blood obtained before burns and obtained at 1 hour and 24 hour intervals following burns from the test animals themselves. No reaction was obtained with either the pre-burn or post-burn specimens. A total of four animals was used for this testing technique. When quantities of serum in excess of 1 cc. were used, a non-specific slow contraction of the muscle was noted. In quantities of 1/2 cc. or less this activity was not present. At no time was the typical anaphylactic response of the muscle noted. Therefore, by this test method, within the limitations of the technique as utilized in our laboratory, autoimmunity to burns could not be demonstrated.

A recent report from the laboratory of Dr. T. G. Blocker from Galveston, Texas, would indicate that repeated burning of animals resulted in a measurable level of antibody in the sera which caused a precipitation reaction with sera obtained immediately

after burns, particularly within 2 to 10 minutes following burns. Because of this demonstration of precipitin activity, it would seem pertinent to repeat the Schultz-Dale guinea pig smooth muscle anaphylaxis test utilizing repeated burns to sensitize the guinea pigs and utilizing serum collected 2 to 10 minutes following an acute burn as the antigenic test agent. These experiments are presently under way.

IV. Preparation of Technique for Measurement of Surface Area and Apparatus for Controlled Thermal Exposure of Laboratory Animals.

In order to provide a source of heat for the burning of animals which would offer consistent and repeatable results, a burn machine was constructed using a gas burner operated on a propane pressure regulated line. A ceramic plate was superimposed so as to give off high intensity infrared radiation when heated by the gas flame. A 5 inch diameter aluminum shutter operating on a solenoid was interposed between the platform for the animal and the burner surface. This shutter is controlled by an electric timing device. Water-cooled aluminum plates were placed in front of and behind the shutter to prevent over-heating and to provide a body surface

temperature of approximately 30°, immediately adjacent to the openings
through which the burn was administered. Calibration of this appara-
tus revealed that .70 calories could be produced per square centi-
meter per second to a blackened surface. A check of this calibration
by means of the infrared sensing device used in the laboratories of
the Medical Field Research Laboratory at Camp Lejeune, showed a
close correlation. With this apparatus the caloric output was
measured at .87 calories per square centimeter per second. At
present the operating area of burn is 25, 2 square centimeters with
virtually no change in intensity of radiation over the entire area.
Exposure times as short as 3 seconds have been shown to cause third
degree burns in the guinea pig. .Graph III demonstrates the working
calibration curve for this machine.

It was also felt important to evaluate the specific surface
area measurements of the guinea pigs in terms of body weight and
to date the only feasible method has been to pelt the animal and
actually measure the area of skin surface from a drawn reproduction
of this pelt.

It has been reported that immersion of an animal into
a water bath which is resting on a scale, up to weights that equal
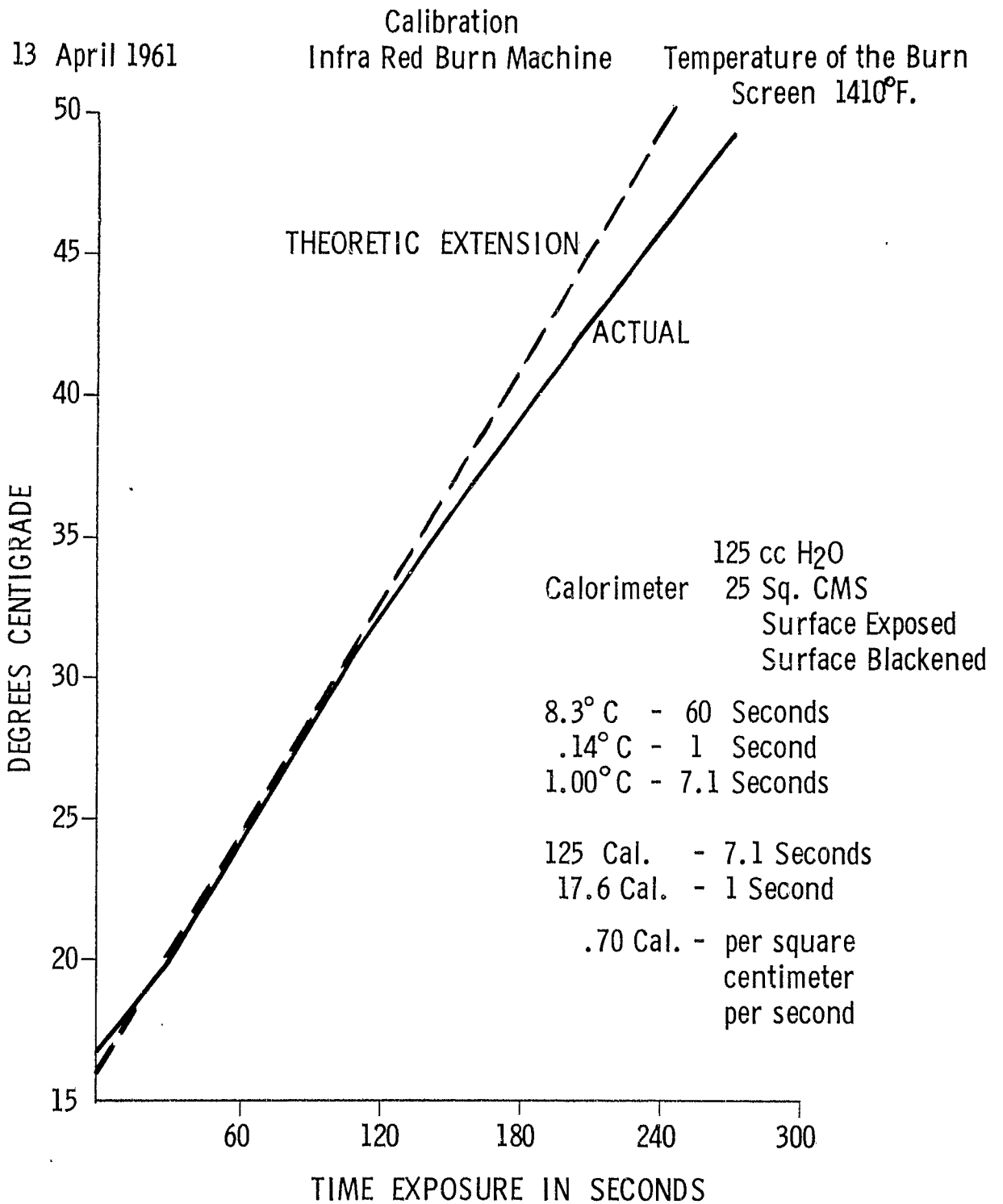various percentages of their total body weight would moisten a skin

GRAPH III

Calibration
Infra Red Burn Machine   Temperature of the Burn
Screen 1410°F.

13 April 1961

THEORETIC EXTENSION

ACTUAL

125 cc H₂O
Calorimeter   25 Sq. CMS
Surface Exposed
Surface Blackened

8.3° C - 60 Seconds
.14° C - 1 Second
1.00° C - 7.1 Seconds

125 Cal. - 7.1 Seconds
17.6 Cal. - 1 Second

.70 Cal. - per square
centimeter
per second

DEGREES CENTIGRADE

TIME EXPOSURE IN SECONDS

TABLE XI

GUINEA PIG AREA-WEIGHT RELATIONS

Guinea Pig #	5% (A)	5% (B)	10%	20%	30%	Weight
73	89.8	90.2	125.2	135.9	187.1	721
74	71.3	80.1	111.6	149.8	201.1	767
77	72.4	67.3	80.5	111.1	133.3	581
78	85.6	82.9	113.4	147.0	194.2	836
79	71.0	75.1	97.8	150.3	174.7	863
80	94.6	98.6	126.2	160.1	190.4	746
82	57.4	82.1	76.6	135.2	-----	563
85	71.2	70.8	102.3	125.0	151.5	502
87	52.3	53.7	67.3	97.7	138.6	496
91	95.7	95.3	136.4	165.6	218.4	948
92	56.3	65.2	82.9	113.4	162.7	765

surface area in square centimeters

area of approximately the same percentage as the percent weight

immersed. To test this hypothesis, 11 animals were immersed in

a dye solution up to 5%, 10%, 20%, and 30% of body weight with the

5% immersion being repeated for purposes of determining accuracy.

The animals were then rolled onto a clean piece of paper so that

the dye would then be applied to the paper and form an outline of

the surface area of the animal which had been immersed. Measure-

ments were then made by means of Keuffel and Esser Planimeter.

As can be seen in Table XI, immersions of from 5 to 20% of body

weights moistened and dyed body surface areas which were not

directly related to the percent weight immersion. At 30% weight

immersion, the body surface immersed, very closely approximated

30% of the total surface area as measured when the animal had been

pelted. Unfortunately the technique does not permit immersion of

more than 30% because of the involvement of the extremities of the

animal giving inaccurate area measurements. At present it seems

best to relate the surface area of the guinea pig directly to the

animal's weight by means of appropriate weight versus area graphs.

It might be feasible to use a weight immersion method to provide

body surface burns in the area of 30% but certainly not 20% or less.

PROPOSED FUTURE WORK

1. The recent visit of Dr. L. Pávková of Prague, Czechoslovakia, to this country and her reports of work with a colloidal gel agglutination test for the detection of autoantibodies to skin in the burn patient, presents the first apparently workable in vitro method for detecting antibodies in burn patients. It is, therefore, proposed that this test technique be attempted in this country. To this end Dr. Pávková has agreed to provide our laboratories with colloidal gel suspension as well as with a number of control positive and negative test specimens so that we may standardize the test technique with those in her laboratory.

It is further suggested that the antigenic material used in Dr. Pávková's test, consisting of homogenized burn epithelial layers, should be evaluated for toxicity in an in vivo system preferably in terms of physiologic changes, or less significantly, in terms of mortality. Thus, an in vivo assay of the benefit of a high titre convalescent sera can be ascertained. In order to approach this work with an open mind we must also include the possibility that antisera developed against one's own skin may not be beneficial to the patient. Therefore, tests of the possible toxic effects of high titre convalescent burn sera will be checked in animals in terms of

rate of re-epithelization of standardized wounds, effect on time of vascularization of standard autograft, as well as effects on the growth of young animals.

2. A tissue culture test to study the motility of cells from ex-plant cultures in the presence of test sera will be attempted on a pilot trial basis with presently available specimens.

3. The guinea pig anaphylaxis test will be carried out utilizing animals which have been subjected to repeated burns in order to produce a maximum immunity response with antigenic challenge by burn blood or serum obtained during the early post burn period from 2 to 10 minutes.

4. Immuno-electrophoresis of acute burn serum and burn skin homogenates will be utilized in an attempt to identify variance in antigenic structure before and after subjection to standard burns.

5. Concomitantly with the above proposed work, a research effort has been organized to identify physiologic changes in urinary function due to burning and to evaluate the effects of convalescent burn serum on these renal functions. This work will be done at Camp Lejeune Medical Field Research Laboratory in conjunction with Lieutenant Coffin of the Physiology Branch and Captain Calvy, Commanding Officer.

DEVELOPMENT OF ANTIBODIES AGAINST TOXIC

SUBSTANCES IN BURNED SKIN

By Franz Schütz
Physiology Dept.   Vienna, Austria

DEVELOPMENT OF ANTIBODIES AGAINST TOXIC

SUBSTANCES IN BURNED SKIN

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Repeated skin burns change the general and local reaction of experimental animals to new burns on previously non-burned areas. Search was made for these toxic substances which develop and are absorbed after repeated burns. Experiments were made with whole blood, with serum and with skin extracts. The experiments were conducted not only to prove toxicity, but to try to find which one of these substances could produce a functionally and anatomically similar clinical picture to burns in other animals. An extract of in vivo burned skin injected into an animal of the same species (no reaction of foreign protein) had a lethal effect and the same well-known pathological changes as in lethal burn. Normal skin extract showed no toxicity under the same conditions.

Burned skin extract very often gave a positive "cutireaction" (0. 1 cc. intracutaneous) in Guinea pigs (sensitized with 1 to 3 skin burns)

whereas normal skin did not. (No signs of inflammation). There was no reaction with either normal or burned skin in non-burned (non-sensitized) guinea pigs.

These substances developed in the burn area do not seem to be species-specific. Skin extract from rabbits, guinea pigs and rats showed practically the same results, using guinea pigs as experimental animal.

With a standard method of burn (same intensity, time and surface area) it could be shown that the different degrees of burn severity were due not only to the burn itself but furthermore to the general conditions of the experimental animal at the time of the burn. Thus the same burn could produce a different degree of burn severity in a normal animal than in a previously burned animal. It could be demonstrated in rabbits that they develop a state of hypersensitivity after a few small area burns, and after repeated burns (every 5 days for at least one month) there is a decreased local and general sensitivity; this could be shown in many but not all experimental animals.

Experiments with Histamine and Histidine showed no evidence of importance in the development of toxicity after burns.

Serum of animals with active immunization (several small burns
at different skin areas and injection of burned skin extract) had a
life saving effect on animals with a usually lethal burn. Control
animals burned to the same extent, injected with normal serum
had no survivors (or only a few survivors).

The local reaction after a "te::t-burn" is usually decreased by an
intravenous injection of specific serum.

SPURIOUS 'AUTO-IMMUNE' REACTIONS IN GEL-DIFFUSION

PLATES

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In work on reactions between mouse tissues and heterologous antisera in gel-diffusion plates a faint line of precipitate sometimes appeared between wells containing normal mouse serum and normal mouse liver or kidney. In view of the possible significance of this finding for work on auto-immune reactions, it was investigated further.

Serum and 50 per cent homogenates of tissue in physiological saline were placed in 2-cm. diameter wells in plates of 1.5 per cent agar. Single precipitation lines were seen after 2-10 days at 4° C., sometimes becoming double or treble later, and optically resembling closely those produced by true antigen-antibody reactions.

The reaction was difficult to study as it varied irregularly in intensity and occurrence, suggesting the operation of an uncontrolled factor. The development of other precipitation lines was a further complication. For example, a band of precipitate, differing from that under study, often developed between sera and various tissues (especially from guinea pigs). It abutted on the tissue-well, was granular in appearance and was found to be microcrystalline.

Reactions were most marked with rat serum, weaker with guinea pig
serum and still weaker with mouse, rabbit and hamster serum. Serum
from rhesus monkeys and pigeons also gave reactions, but these were
not extensively investigated. Rat tissues gave the most intense reactions
and then, in order of intensity, guinea pig, rabbit, mouse and hamster
tissue.

In the rat, reactions were given by kidney, liver, adrenals, brain,
stomach, small gut, spleen, heart, lung and skeletal muscle; and
were faint or absent with washed erythrocytes, thymus, testis,
seminal vesicle, ovary and uterus. The lines produced by different
tissues gave a reaction of identity.

Serum from any individual reacted with its own tissues as well as
with those from other individuals, and serum from one species reacted
with the tissues of other species as well as with those of its own. The
reaction was not dependent on sex.

The reaction could be carried out with serum diluted up to 32-fold
and with tissues diluted up to 8-fold. It was best effected at 4° C.,
was weaker at room temperature and absent at 37° C. Precipitation
lines were marked in agar made up in 0·05 M phosphate buffer,
distilled water or 1 per cent sodium azide, were weaker in the presence

of 0·14 M sodium chloride and absent at concentrations of this salt greater than 2 per cent. Gels at pH between 5·5 and 9·8 could be used satisfactorily.

The heat-lability of the substances responsible for the reaction varied considerably, but usually the serum factor was destroyed after 1 hour at 56° C. and the kidney factor after 4-5 hr. at this temperature. The liver factor was more heat-resistant and often showed doubling or trebling of the precipitation line after heating. Both serum and tissue factors resisted freeze-drying, passed Seitz filters and were not removed by dialysis against 0·14 or 2 M saline. Exposure to a pH of 4 or less, or 12 or more, for 1 hr. destroyed the serum factor. The corresponding pH's for the kidney factor were 3 and 10. The serum factor was precipitated by 50 per cent saturation with ammonium sulphate.

The reaction thus differs from that reported by Korngold et al.[1], which was not given by undialysed serum, and from the reactions of Tomasi, and of Metzgar and Grace[2], in that their serum factor was identifiable as serum albumin. It also differs from that between red cell lysates and serum, reported by Peetoom et al.[3], in its occurrence at alkaline pH and its abolition by high salt concentration. When

the tissue-serum reaction described here and the haemolysate-serum reaction of Peetoom et al. [3] were effected in the same gel, the respective precipitation lines crossed without reaction of identity.

Staining and extraction tests were carried out on the lines of precipitate, with the results shown in Table 1. The precipitate appears to contain ribonucleic acid. The effect of sodium chloride suggests that the reaction involved a salt-like combination, perhaps between a basic protein and ribonucleic acid.

These and other findings [1-3] show that diffusible components of normal tissues can react in gels to form precipitates superficially resembling those produced by true antigen-antibody reactions, a fact to be borne in mind in work on the immunology of tissues.

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